DEVELOPMENT OF AN OPTICAL IMMUNOSENSOR BASED ON THE EVANESCENT WAVE TECHNIQUE

A dissertation submitted for the degree of

Doctorate of Philosophy

by

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Declaration

I hereby certify that the material, which I now submit for assessment on the programme of study leading to the award of Ph.D, is entirely my own work and has not been taken from the work of others save, and to the extent that such work has been cited and acknowledged within the context of the text.

Signed: Teresa Mc Cormack Date: 14 July '95

Teresa A. Mc Cormack

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Abstract

The research centered on the development of an antibody-based optical biosensor. The enzyme lactate dehydrogenase was chosen as the analyte, as it is a clinically important enzyme and is used as an indicator of various disease states such as heart disease.

Polyclonal antibodies were raised in rabbits. These antibodies were purified, characterised and labelled with horse radish peroxidase and fluorescein. Enzyme-linked and fluorescent-linked immunoassays were developed using the HRP-labelled and FITC-labelled antibodies, respectively.

Antibodies specific for chicken heart lactate dehydrogenase (CHLDH), were immobilised on the exposed core of optical fibres. The immobilisation procedure was optimised to ensure reproducibility of immunoglobulin immobilisation. An optical immunosensor, based on the evanescent wave technique, was developed and optimised. The sensor was shown to be specific for CHLDH and sensitive to varying concentrations of CHLDH.

A comparison was made between one-step and two-step assay systems using the evanescent wave immunosensor and the BIAcore (commercially available biosensor based on surface plasmon resonance). The sensors were also compared in an experiment to determine the effect of high levels of fluorophore labelling on antibody affinity.

Anti-human H_4LDH (LDH_1 isoenzyme) antibodies were also raised and tested in an ELISA system using human serum samples. The isoenzyme profiles of these serum samples were also prepared to see if elevated LDH_1 (H_4LDH) levels could be used as an indicator of myocardial infarction. The specificity of the antibodies was checked using immunoblotting techniques.

A minituarised colourimetric assay for total LDH was developed and validated using patient samples.

Abbreviations

Ab	antibody
Abs	absorbance
Ag	antigen
AMI	acute myocardial infarction
BCA	bicinchoninic acid
BSA	bovine serum albumin
°C	degrees Celcius
CHLDH	chicken heart lactate dehydrogenase
Ci	Curies
cm	centimetre
conc	concentration
CV	coefficient of variation
(k)Da	(kilo)Daltons
DMF	dimethylformamide
DMSO	dimethylsulphoxide
EDC	N-ethyl-N'-(dimethylaminopropyl) carbodiimide
EDTA	ethylenediaminetetra-acetic acid
ELISA	Enzyme-Linked ImmunoSorbent Assay
EWS	evanescent wave sensor
Fc	constant portion of IgG molecule
FITC	fluorescein isothiocyanate
F/P	fluorophore-to-protein ratio
g	gram
GMBS	N-gammamaleimidobutyryloxy succinimide ester
h	hour
H_4	LDH isoenzyme 1 (i.e. 4 H subunits)
HBS	Hepes buffered saline
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
Hz	Hertz
IgG	immunoglobulin class G
IgM	immunoglobulin class M

L	litre
LDH	lactate dehydrogenase
LDH_x	LDH isoenzymes 1,2,3,4 or 5
LED	light emitting diode
LIA	lock-in amplifier
log	logarithmic
m	metre
M	molar
M_4	LDH isoenzyme 5 (i.e. 4 M subunits)
max	maximum
mg	milligram
min	minute(s)
ml	millilitre
mm	millimetre
mM	millimolar
mol	moles
MTS	mercaptopropyltrimethoxysilane
m.w.	molecular weight
mV	millivolts
µg	microgram
µl	microlitre
µM	micromolar
µV	microvolts
N.A.	numerical aperture
NAD	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NC	nitrocellulose
NHS	N-hydroxysuccinimide ester
nm	nanometre
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PC	personal computer
PDA	photodiode array
PEG	polyethylene glycol

PMT	photomultiplier tube
r	regression coefficient
rpm	revolutions per minute
Rt	retention time
RT	room temperature
RU	response units
SDS	sodium dodecyl sulphate
sec, s	seconds
SpA	protein A
SPR	surface plasmon resonance
UV	ultraviolet
V	volts
v/v	volume per unit volume
w/v	weight per unit volume

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CHAPTER 1

INTRODUCTION

1.1 Introduction

A sensor is a device capable of providing a usable electrical signal output in response to the continuous recording of a physical parameter or the concentration of a specified chemical or biochemical species. The output signal is processed and displayed in a convenient format. The measurand is generally inherently different from the desired output signal and so at least one transduction step is required to convert the measurand quantity into an electrical signal, for the sensor to work. Biosensors are a subset of sensors, which employ the specificity of biological molecules, e.g. enzyme, antibody, cell etc., responsive to the property or analyte being determined, in contact with a suitable transducing element, that converts the signal sensed into a signal that can be conveniently processed (Vadgama and Crump, 1992). A schematic diagram of the biosensor set-up, is shown in Figure 1.1. The signal sensed is a result of a change in one or more physico-chemical parameters associated with the bioreaction, and manifests itself as the production of ions, electrons, gases, light, heat or mass (Hall, 1990).

The analysis of biochemical compounds, in relation to specific identification and the determination of their concentrations is a major activity all over the world. Medical diagnostics (Connolly, 1995), healthcare (Owen, 1994), agriculture, veterinary science, industrial process control (Lüdi *et al.*, 1991) and environmental monitoring (Dennison and Turner, 1995) are a few of the real life situations where analytical information is required every day. Any analytical device which would simplify the analytical methodologies employed, to a level where they could be routinely used in decentralised environments, by relatively unskilled operators, offers many advantages.

This was the rationale for combining expertise from many diverse disciplines including biochemistry, electrochemistry, optronics, physics and electronics, with the strategic aim of developing 'biosensing devices'.

The first biosensor was described by Clark and Lyons (1962). The 'enzyme electrode' biosensor consisted of a platinum electrode which responded to peroxide generated by the following reaction;

$$\text{glucose} + O_2 \xrightarrow[\text{oxidase}]{\text{glucose}} \text{gluconic acid} + H_2O_2$$

Here, the substrate glucose, was acted upon by the enzyme glucose oxidase, to give rise to gluconic acid and peroxide. The amount of peroxide generated was proportional to

the concentration of glucose and, hence, the system could be used to monitor glucose levels.

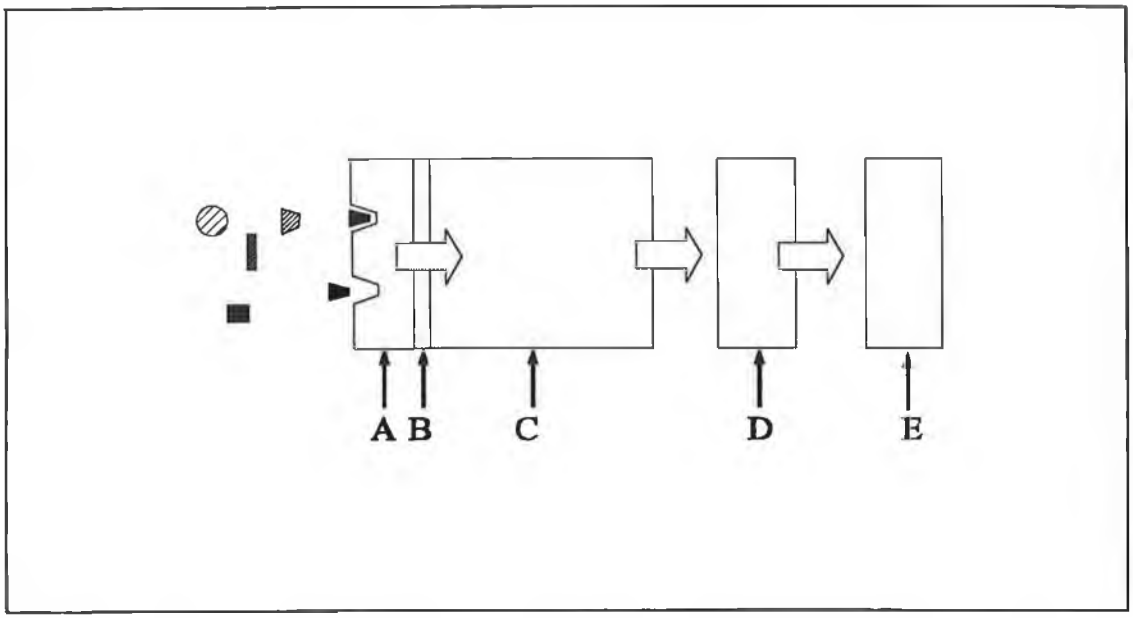


Figure 1.1: Schematic of biosensor set-up. The biosensor has three main parts. The *biological entity* **(A)** normally interacts specifically with the analyte in the sample, resulting in a physico-chemical response **(B)**. The *transducer* **(C)** converts the physico-chemical response resulting from the binding of the analyte with the biological entity, into an electrical signal. This electrical signal is converted by the *output device* **(D)** into a format that may be monitored by the user via a signal display **(E)**.

Biosensors have great potential for rapid analysis with the ability to determine specific analytes in complex environments. In some cases, the biological reaction is detected as it happens and the biosensors are said to operate in 'real-time'. Ideally, a biosensor should eliminate the need for sample pretreatment, be easy to use, be of compact transportable size, allow one step reagentless analysis and have a rapid response turn-around time (Figure 1.2). Such devices would allow analytical measurements to be taken *in situ*, giving information on dynamic processes which could be responded to, immediately, e.g. monitoring of blood glucose in diabetic patients (Greenough *et al.*, 1994).

The commercialisation of biosensors is hindered by practical problems which must be overcome, such as the stringent operational requirements of biomolecules which put limitations on biosensors (Griffiths and Hall, 1993). Also, the bioelement must come

into direct contact with the test solution which may result in non-specific interactions with the transducer surface. Biological components are also inherently unstable and, therefore, operational life-times of biosensors tend to be short (Scheller *et al.*, 1991).

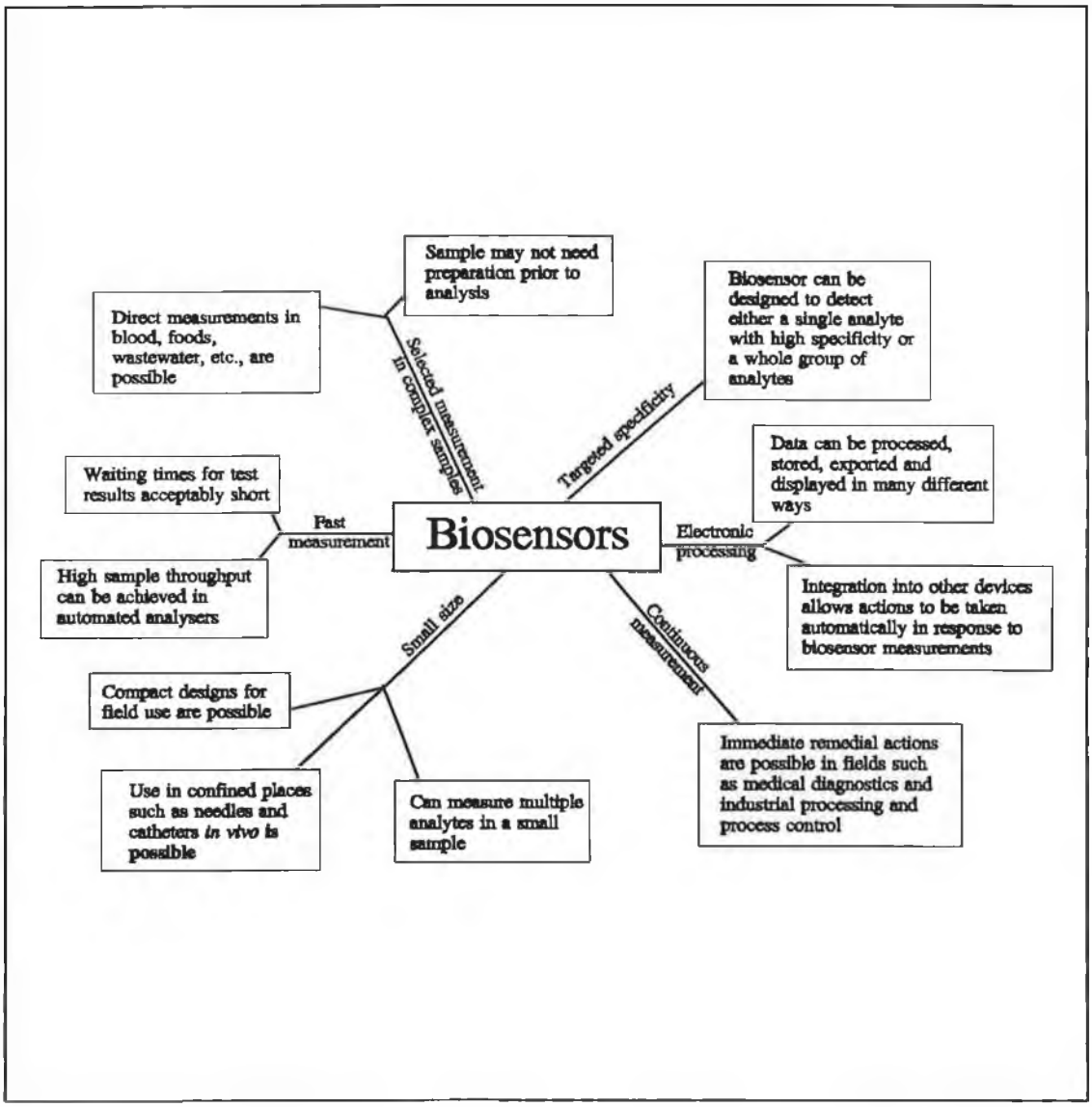


Figure 1.2: Characteristics of an 'ideal' biosensor (Taken from Griffiths and Hall, 1993).

1.2 The Biological Component

Many key reactions in biological systems are based on recognition phenomena (enzyme-substrate, antibody-antigen, receptor-ligand), and it is the specificity of these recognition events in which lies the advantages of biosensors. Biosensors can be divided into two main groups: *catalytic* and *affinity* biosensors, depending on the biological molecule employed, but in each type, a recognition event is involved (Byfield and Abuknesha, 1994). In catalytic biosensors, the biomolecule recognises its analyte and changes it in some way, and it is a change in the concentration of some species involved in the catalytic reaction that is detected. With affinity biosensors, however, the binding reaction itself is monitored, either directly through some subtle charge effect, conformational or mass change, or indirectly, using a reporter molecule, such as an enzyme or fluorophore. Indirect methods are more common as direct methods of monitoring are often difficult to transduce (Leech, 1994).

1.2.1 Catalytic biomolecules used in biosensors

1.2.1.1 Enzyme-based systems

The most commonly employed biological component in catalytic biosensors is an enzyme. Enzymes are routinely used as analytical tools outside the body due to the numerous, diverse reactions they catalyse. They include oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases. Oxidoreductases act by transferring electrons or hydrogen ions during the oxidation and reduction of substrates and so are particularly suitable for use in electrochemically-based biosensors.

Cass *et al.* (1984), described an amperometric-based biosensor, for the determination of glucose in whole blood samples. It was later commercially developed by MediSense (Cambridge, MA, USA) and collaborators at Oxford and Cranfield (England), into the ExacTech pen-type glucose sensor, for monitoring glucose levels in diabetic patients. The biosensor employed the oxidoreductase enzyme, glucose oxidase and the soluble mediator ferrocene (bis(η^5-cyclopentadienyl)iron), $Fecp_2R^+$. Direct electron transfer between the biomolecule and the electrode surface is obtainable but only if there is extremely short distances between the redox site within the protein and the electrode surface (Armstrong *et al.*, 1988). For large proteins, the redox site may be buried deep within the protein and may not be accessible. A mediator is a low molecular weight redox couple, which can shuttle electrons from the redox centre of the enzyme to the

surface of the sensing electrode. The general reaction scheme for the bioreaction and electron transfer is;

$$\text{glucose} + \text{GOD(ox)} \longrightarrow \text{gluconolactone} + \text{GOD(red)}$$

$$\text{GOD(red)} + 2\text{Fecp}_2\text{R}^+ \longrightarrow \text{GOD(ox)} + 2\text{Fecp}_2\text{R} + 2\text{H}^+$$

$$2\text{Fecp}_2\text{R} \longrightarrow 2\text{Fecp}_2\text{R}^+ + 2e^- \longrightarrow \text{electrode current}$$

The enzyme glucose oxidase, GOD, is co-immobilised with the mediator, $Fecp_2R^+$, on the surface of the amperometric electrode. On converting the substrate glucose to its product, gluconolactone, the enzymes prosthetic group, FAD, is reduced to $FADH_2$. Normally, the enzyme would be reoxidised by molecular oxygen present in the sample, but in this case, the mediator accepts the electrons, to reoxidise the enzyme. The mediator itself is reoxidised by surrendering the same electrons to the electrode surface, resulting in a faradic current, which is directly proportional to the concentration of glucose in the sample.

1.2.1.2 Whole cell and tissue-based systems

One of the principle problems associated with enzyme-based biosensors is stability. Many enzymes are extremely sensitive to pH and temperature, particularly in their purified form. The use of intact microbial or mammalian cells (tissue slices), rather than purified proteins, offers many advantages (Scheller and Schubert, 1992). Whole cells are sources of undamaged stable enzymes in their natural, fully optimised environment, and are inexpensive when compared to purified enzymes. Complex catalytic reactions which are difficult or impossible to reproduce outside the cell with purified enzymes, can be exploited in whole cell systems, but conversely the response may not be specific due to the plethora of enzymatic pathways present. The effect of an analyte on the overall metabolism (inhibition of growth, cell viability, substrate uptake, respiration) of the cell can also be determined (Karube and Nakanishi, 1994). Whole cell biosensors do have the disadvantage of high diffusional resistance of the cell, in that the substrate must diffuse in and the product out of the cell, and must then diffuse to the transducer surface before it can be detected. This effectively increases the response time of the sensor.

Transducers used to create biosensors based on microbial reactions include potentiometric systems (ion-selective electrodes, pO_2-, pCO_2- and pNH_3-electrodes), thermistors, photometers and piezoelectric membranes. Oxygen-sensing electrodes have been most commonly used, due to the large number of sensing systems based on the

metabolic activity of the microorganism. Increases in respiratory activity are normally caused by the assimilation of substrates (analytes) and is recorded as a decrease in O_2 tension.

The biochemical oxygen demand (BOD), is one of the most widely used and standard tests for the measurement of organic pollution in water. The main drawback of conventional BOD measurement is that it requires a five day incubation period. A rapid and more reproducible method is therefore required for measuring BOD. Biosensors employing microorganisms have been described for measuring BOD (Karube and Nakanishi, 1994). One example was reported by Tan *et al.* (1993). Their sensor consisted of a membrane containing the immobilised microorganisms, *Bacillus subtilis* and *Bacillus lichenformis*, to effect the biooxidation process, and an electrochemical oxygen probe. The selectivity of these microorganisms is low in that they have high biooxidation activity for a wide range of organics. The ability of the mixed microbial culture to biodegrade organics resulting in the consumption of oxygen due to microbial respiration, was measured by the Clark oxygen electrode (Clark and Lyons, 1962). This biosensor could indicate the level of pollution from the BOD in 15-30 seconds with a baseline recovery time of 4 minutes, using initial rate measurements, compared to the conventional methods which takes up to five days.

A "BOD-module" produced by Medigen (Prufergate-Werk Medigen GmBH, Germany), measures BOD in the range 2-22 mg/L in a response time of 50 seconds. Each membrane of immobilised microorganisms is viable for 2,000 or more assays, since the biocatalytic activity can be replenished simply by placing the electrode in nutrient media (Dennison and Turner, 1995). New cells grow *in situ*. However, due to the build up of dead cells, the sensor eventually must be discarded.

Whole mammalian tissue slices or cells offer similar advantages to those provided by microbial systems (Wijesuriya and Rechnitz, 1993). Tissue slices can easily be immobilised to transducer surfaces by simple mechanical fixation using a semi-permeable membrane or nylon net. They can be additionally crosslinked for mechanical stability. Tissue slices and purified enzymes can be co-immobilised thereby increasing the diversity of such biosensors.

An example of a tissue-based biosensor was reported by Xiuli *et al.* (1992). Rabbit thymus tissue contains a high concentration of adenosine deaminase which catalyses the release of NH_3 from adenosine, and therefore can be employed for the quantitation of adenosine in body fluid samples. Xiuli *et al.* (1992), immobilised a slice of rabbit

thymus tissue on a monofilament nylon mesh with glutaraldehyde, which was in contact with a NH_3-gas sensing electrode. This sensor yielded an excellent electrode response for the quantitation of adenosine in body fluid samples, without sample processing. The sensor also had a longer lifetime than earlier biosensors, with the additional advantages of simplicity, lower cost and time saving.

Mammalian cells cultured *in vitro* can be used as the biological sensing element. For example, Nakamura *et al.* (1993), developed a rapid detection system for allergic reaction using rat basophilic leukemia (RBL-1) cells as the biological element and cyclic voltammetry as the transducing mechanism. The cultured cells were immobilised to a membrane by filtration. When DNP was added to anti-DNP IgE sensitised RBL-1 cells, it triggered them to secrete serotonin and histamine, which participate in a variety of acute allergic and inflammatory reactions. Serotonin present in the cell is electrochemically oxidised and measured by cyclic voltammetric techniques. The peak current increased linearly with increasing allergen (DNP) concentration within 20 minutes. Such a sensor would be impossible without the use of whole living cells.

Plant tissues are also effective catalysts as a result of the enzymatic pathways they possess, and are inexpensive compared to mammalian cell systems. Banana pulp tissue immobilised on the gas permeable membrane of a Clark-type oxygen electrode was used to measure dopamine, via the enzyme polyphenol oxidase (Sidwell and Rechnitz, 1985).

1.2.2 Affinity biomolecules used in biosensors

1.2.2.1 Antibodies and antigens as the biological component

Many biospecific recognition systems and interactions take place reversibly and without analyte conversion, i.e. in a non-destructive fashion. Antibody-antigen interactions are well documented (Jefferis and Deverill, 1991). The advantage of the immunoassay as an analytical system is that antibodies can be prepared against almost any molecule, whether naturally occurring or not, thus increasing the diversity of immunosensor applications. With the advent of the combinatorial phage display system, the entire immunological repertoire of an animal can be cloned (Barbas *et al.*, 1991). That is to say that the all the possible combinations of antibodies produced by the body can be generated. Analytes as varied as food constituents, microbes, enzymes, drugs, hormones, tumour associated antigens, pesticides, and other pollutants can be measured employing antibody-based techniques (O'Kennedy, 1989). Some immunoassay formats are detailed in chapter 3.

Since the physicochemical changes caused by immunoreactions are very slight, in many cases reporter molecules coupled to the antibody or antigen may be required depending on the transducing mechanism. A major drawback of immunosensing is reusability. Antibody-antigen dissociation kinetics are very slow and repeated regeneration by dissociation of the antibody-antigen binding, often results in denaturation of the immunoreactants (Miller and Anderson, 1989). Low affinity monoclonal antibodies may be selected, but this may compromise the sensitivity of the sensor.

Examples of antibody-based biosensors will be discussed in section 1.4, under the various transducing principles.

1.2.2.2 Receptors as the biological component

Some affinity-based sensors are based on cell receptors. Mammalian cells utilise receptors for the transmission of signals across the lipid bilayer membrane which separates intracellular and extracellular regions of the cell. *In vivo*, the binding of a ligand to its receptor, initiates a particular physiological response in the receptor-bearing organism, through activation or blockage of a series of biochemical reactions (Straub and Bolis, 1978). Such events include hormone regulation, viral replication and neural transmission. Ligands can be as varied as neurotransmitters, amino acids, steroid hormones, metal ions, hormones, toxins, etc (Taylor and Kennewell, 1993).

An example of such a ligand-receptor interaction is the binding of acetylcholine, a neurotransmitter, to its receptor in the sensitive ending of postsynaptic neurons, stimulating them to transmit an impulse. In their isolated form, receptors can be used in biosensor devices, based on the specific recognition of the ligand for its receptor, in a similar manner to immunological reactions. Purified receptors, however, generally have stability problems, and are often labile at room temperature (Leech, 1994). Some receptors such as cholinergic receptors require the presence of phospholipids in order to function, and so must be reconstituted using phospholipid bilayers (Taylor, 1991) for use in biosensors.

An alternative approach, is to use the receptors in their natural form, as intact tissue slices or in isolated whole cells. Such intact biological systems, allow the ligand-receptor binding reaction to be transduced directly by the biological component. In contrast to most biosensors, in which only the molecular recognition element is biological, the antennular receptrode of the blue crab, uses biological components as both molecular recognition elements and transducers (Buch and Rechnitz, 1989). A disadvantage of such

systems is that tissue extracts may contain receptors with other binding specificities, thereby reducing selectivity. Belli and Rechnitz (1986), used the antennular structures from blue crabs which were sensitive to amino acids in solution and the concentration of the amino acids were determined by the frequency of the potential spikes on an oscilloscope. Other examples of receptor-based biosensors, are discussed in section 1.4.

1.2.2.3 DNA as the biological component

DNA is a double helix consisting of antiparallel strands in which the nucleotide units are linked by phosphodiester bonds. The backbone provides the exterior and purine and pyrimidine bases are stacked in the interior in pairs, in which A is complementary to T while G is complementary to C. The strands separate and use complementary base pairing to assemble daughter strands in a semi-conservative fashion.

The blue print for every cell is encoded in its DNA in the form of a series of base sequences. Interchain binding in nucleic acids is extremely specific and recognition of these sequences is of fundamental importance in the control, reading and detection of this information (Lewin, 1990). The property of base complementarity makes possible the construction of DNA probes, which can be used for analytical techniques. If the sequence of bases comprising a certain part of the DNA is known, then the complementary sequence can be synthesised. This sequence is called a DNA probe.

There is increasing awareness of the importance of genetic factors in diseases. DNA can be used as the sensing molecule in biosensors in the detection of, for example, mutations causing a particular disease. Target sequences for a particular gene (or mutated gene), can be synthesised by molecular biological (polymerase chain reaction) or chemical methods, thus supplying the probe. The probe can then be labelled with a reporter group to facilitate transduction.

One example of a DNA-based biosensor was described by Fawcett (1987). The piezoelectric DNA-based biosensor measured the DNA of the pathogen *Salmonella typhimurium* in food. No reporter molecule was required in this case as the binding reaction is transduced as a mass change, which alters the resonance frequency of the crystal.

A fibre-optic evanescent wave biosensor based on DNA intercalation was described by Pandey and Weetall (1995). Organic carcinogens, certain toxins and anti-tumour drugs intercalate with DNA. Double-stranded DNA was immobilised to the fibre surface and fluorescent ethidium bromide allowed to intercalate with the DNA, resulting in a

fluorescent signal. There was a decrease in the response of the sensor when other DNA intercalators were reacted with the fibre probe, as they compete for the intercalation sites on the dsDNA, displacing ethidium bromide.

1.2.2.4 Other biological binding molecules as the biological component

There is also a class of binding proteins which are not strictly biological receptors. Binding pairs such as avidin-biotin (Wilchek and Bayer, 1988), protein A-IgG (Surolia *et al.*, 1982), yeast mannan-concanavalin A (Janata, 1975), represent such interactions. These binding pairs of molecules have limited but useful interactions. They are more robust than biological receptors as regards immobilisation and therefore adsorption and covalent binding are usually employed (Taylor, 1991).

1.3 Immobilisation of biomolecules for biosensors

The biosensing surface of biosensors is made up of enzymes, antibodies, antigens, microorganisms, mammalian cells, tissues or receptors immobilised on to a solid surface. There are a number of established procedures for immobilisation (Figure 1.3), which include;

a) physical adsorption on to a solid surface

b) use of cross-linking reagents,

c) entrapment using a gel or polymer,

d) use of membranes to retain the biomolecule close to the electrode surface,

e) exploitation of biomolecular interactions, e.g. avidin/biotin, protein A/Fc domain of class G immunoglobulins,

f) covalent attachment.

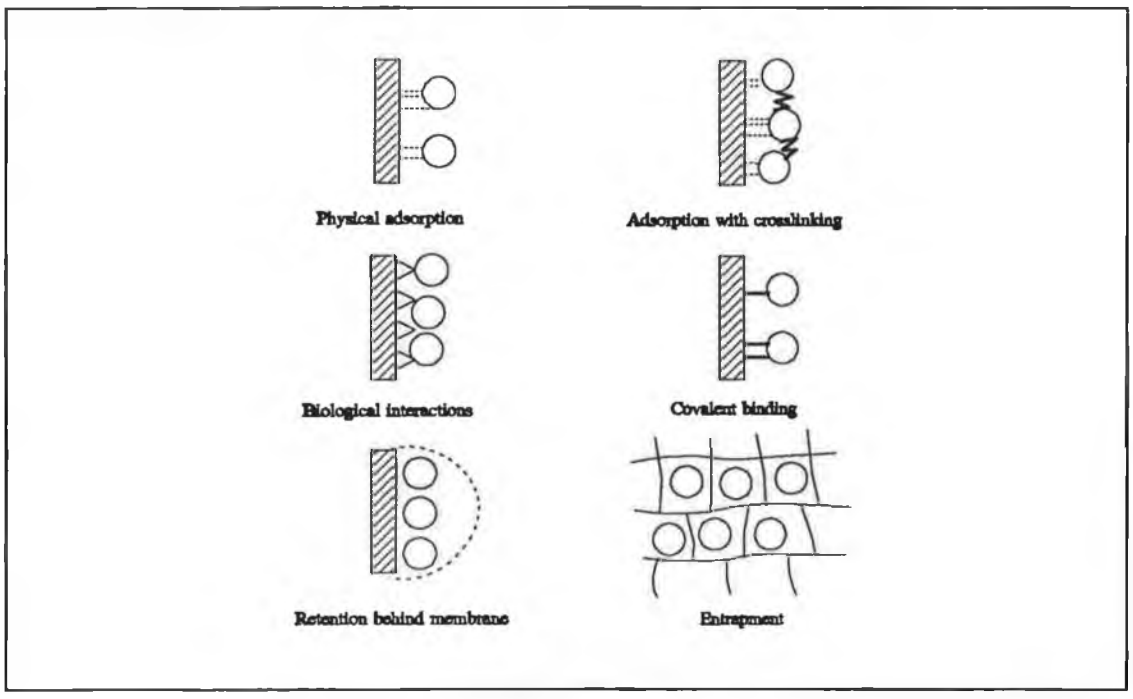


Figure 1.3: Methods of biomolecule immobilisation for biosensors

The choice of immobilisation method, depends on the biocomponent to be immobilised, the nature of the solid surface and the transducing mechanism. Whatever procedure is used, it must take into account the following factors;

a) the activity of the biomolecule must be preserved and the specificity must not be reduced,

b) the stability of the biomolecule should be maintained or preferably increased. This is important in relation to the stability of the sensor system for use, reuse and storage,

c) steps should be taken to ensure that no non-specific binding can occur as a consequence of the immobilisation procedure,

d) ideally, the methods used should be easy to perform and be highly reproducible, to facilitate mass production of the sensor device,

e) the immobilisation procedure should not involve extreme conditions of pH, temperature, salt concentration etc., which may adversely effect the activity of the immobilised biomolecule.

It should be noted, however, that many biomolecules, given the correct conditions, can be subjected to relatively high temperatures, for short periods of time. By using biomolecules from thermophilic organisms, or by using chemical and genetic modification, it should be feasible to produce 'designer' molecules capable of being reactive, under defined conditions (Ó Fágáin and O'Kennedy, 1991). This area is now under investigation.

Some of the immobilisation methods for biosensing devices and the advantages and disadvantages of each are outlined in Table 1.1.

Physical adsorption: Immobilisation via physical attraction or adsorption is not a reproducible and reliable method of biomolecule attachment to sensing surfaces because of the problems associated with leaching during long term storage. Plastic, glass and cellulose have been known to adsorb proteins, via binding forces such as hydrogen bonds, van der Waals forces, salt linkages and hydrophobic interactions. With the exception of hydrophobic interactions, such forces are not very stable and can easily be disrupted by changes in pH, temperature and ionic strength. Excess protein can also form multiple layers during adsorption. These layers are not very stable and easily desorb. The obvious advantage of adsorption is its simplicity and gentleness, although some loss in activity is observed. However, in the case of antigens, care should be taken to ensure that the antigenic binding sites are not denatured in any way.

Use of crosslinking reagents: Stabilisation of adsorbed proteins can be achieved by using bifunctional reagents such as glutaraldehyde. The proteins are crosslinked to each other, or inert proteins, such as bovine serum albumin, can be mixed with the desired protein prior to crosslinking. This adds greater stability to the immobilised protein although inevitably, some inactivation does occur, as the crosslinking chemical may interact with the active site of the biomolecule. With respect to immunosensors, the antibody binding site may be blocked or incorrectly orientated, so as not to favour antibody-antigen binding. Membranes can be cast on electrode surfaces using this method.

Entrapment: Immobilisation of biomolecules by physical entrapment in gel matrices such as polyacrylamide or gelatin, is a mild method of immobilisation. Basically, the polymer is allowed to crosslink in the presence of the substance to be immobilised. The porosity or degree of crosslinking can be controlled. Evidence has shown that polyacrylamide gels give the best retention of enzyme activity. Care should be taken however that the crosslinks retain the molecule, and that leaching does not occur. Microorganisms and antibodies are often immobilised by physical entrapment.

Use of membranes to retain the biomolecule close to the transducer surface: Membranes of various porosities can be used to retain molecules close to transducer surfaces, without the need for actual immobilisation. The gentle method of retention can, however, lead to problems such as diffusional resistance. 'Selective' membranes can also be employed, in conjunction with potentiometric electrodes. Ion-selective membranes, such as an NH_4^+-selective membrane, has been employed in conjunction with the enzyme urease, for the measurement of urea (Petersson, 1988).

Exploitation of biological interactions: The ability of protein A to bind to the Fc portion of class G antibodies has been exploited for antibody immobilisation. Such an interaction allows the antibody to be orientated so as to favour antibody-antigen interactions. The protein A molecule must first be immobilised by typically covalent methods, however. Other biological interaction immobilisation systems which have been exploited include the avidin-biotin system. Such systems, however, are limited by problems due to leaching and expense. The cost of purified preparations of these binding molecules is very high.

Covalent attachment: Chemical coupling of biomolecules may provide stable biosensing surfaces which are resistant (in terms of leaching), to wide ranges of pH, temperature and ions. Covalent binding can result in some loss in bioactivity. Three types of supports have been used: inorganics, natural polymers, and synthetic polymers. The binding process must occur under conditions which do not denature the biomolecule. Often, the carrier must be activated in some way (e.g. silanisation), and the introduced functional groups are then utilised for chemical coupling either directly to the biological molecule or via a crosslinking reagent such as glutaraldehyde. Alternatively, nylon nets are activated by methylation, and treated with lysine, before the required biomolecule is covalently linked to the net. The nylon net disc is then simply fixed onto the sensor surface. The biological component of amperometric biosensors is often immobilised covalently for efficient transfer of redox species to the electrode surface.

The area of enzyme, antibody and receptor immobilisation for biosensors is discussed in excellent reviews by Guilbault *et al.* (1991) and Taylor (1991).

1.3.1 Immobilisation of receptor molecules

Receptor-based biosensors provide a unique capability for diagnostics and detection enabling the detection of an unknown analyte based on its biological activity. The immobilisation of cell-membrane receptor proteins requires special consideration. Studies have shown that if purified receptor proteins (such as adrenergic, cholinergic and opiate receptors), are reconstituted in lipid layers, such as bilayer lipid membranes, phospholipid vesicles and liposomes, many of them retain their functionality (Taylor, 1991). These lipid layers have added stabilisers, such as membrane structural components and detergents. Gotoh *et al.* (1987), showed that the response of an ISFET sensor was increased threefold, when the acetylcholine receptor was reconstituted in a lipid membrane compared to when it was immobilised directly to the transducer surface. A practical biosensor employing lipid membranes is unlikely, however, due to the poor stability of the lipid membranes.

Studies are continuing into other methods of stabilising receptor proteins using non-lipid layer immobilisation technology. Albumin-based membranes stabilised with detergents, lipids and anti-oxidants, have been shown to stabilise acetylcholine receptors with complete retention of biological activity, for at least 6 months, whereas non-immobilised receptors lost all activity after 15 days storage (Taylor, 1988).

Method	Advantages	Disadvantages	Ref.
Entrapment into/by membranes and films coating or associated with a transducer	Non-chemical treatment; mild reaction conditions	Leakage possible; membrane/film may limit analyte diffusion	Aubeck *et al.* (1993)
Entrapment into a lipid bilayer or a liposome coating or associated with a transducer	Non-chemical treatment; mild reaction conditions; mimics natural membranes	Leakage possible; very susceptible to changes in pH, temperature and ionic conditions	Gotoh *et al.* (1987)
Adsorption directly to the transducer or a film/membrane on the transducer	Non-chemical treatment; mild reaction conditions	Leakage possible; very susceptible to changes in pH, temperature and ionic conditions	Rogers *et al.* (1991)
Biological binding to a specific molecule already immobilised on the transducer	Strong, reversible, non-covalent binding; mild reaction conditions	Leakage possible; binding site for matrix must be different from the analyte site	Colapicchioni *et al.* (1991)
Cross-linking into membranes/films	Mild reaction conditions; high analyte permeability; can mimic natural membranes	Possible loss of activity	Boitieux *et al.* (1989)
Covalent binding directly to the transducer	Strong bond resistant to large changes in pH, ionic strength and temperature	Possible loss of activity; transducer recycling unlikely	Shriver-Lake *et al.* (1993)
Covalent binding to a membrane/film on the transducer	Strong bond resistant to changes in pH, ionic strength and temperature; transducer may be recycled	Possible loss of activity; stability may depend on membrane/film-transducer bond	Minunni and Mascini (1993)

Table 1.1: Biomolecule immobilisation methods for biosensors (Adapted from Taylor, 1991).

1.4 Transduction

In a biosensor, the function of the transducer is to convert the physicochemical change associated with the specific and reversible interaction of the biologically active material with the analyte, into an electrical signal, which can be further amplified, processed and displayed. The physiochemical change may be the production of ions, gases, electrons or a change in mass, light or heat. The three main types of transducers, electrochemical, optical and piezoelectric, will be discussed here, with reference to affinity-based biosensors.

1.4.1 Electrochemical systems

1.4.1.1 Amperometric affinity-based biosensors

Amperometric sensors operate on the principle of applying a constant potential between a working and auxiliary electrode in a test environment, which causes non-spontaneous electron transfer to occur. The potential chosen can selectively determine which species are made electroactive. A steady state current results from the electron transfer reaction which is directly and linearly proportional to the concentration of the electroactive species. A reference electrode of fixed potential is also required.

Amperometric biosensors are generally enzyme electrodes. Oxidoreductases, which catalyse oxidation and reduction reactions by the transfer of hydrogens or electrons, are the most commonly used in amperometric biosensors. Affinity-based sensors are generally based on analytical systems involving enzyme (usually of the oxidoreductase class) labelling of one of the binding partners, e.g. antibody or antigen. Amperometric biosensors are very prone to protein fouling of the surface at the electrode and hence the biological molecules are generally immobilised on selectively permeable membranes (Vadgama and Crump, 1992). These membranes prevent electrode fouling, control mass transport of the substrate and retain the bioactive layer.

An example of an affinity-based amperometric biosensor employing an enzyme label for indirect transduction of the binding event was described by Manning *et al.* (1994).

The amperometric immunosensor was based on the catalytic effect of horseradish peroxidase on the reaction between hydrogen peroxide and hydroquinone.

$$H_2O_2 + HQ \longrightarrow 2H_2O + BQ$$

$$BQ + 2H^+ + 2e^- \longrightarrow HQ \text{ (applied voltage -0.03V)}$$

The antigen inhibition competitive assay for factor VIII (a glycoprotein that is quantitated in the diagnosis of haemophilia), was performed in a batch mode of operation. Factor VIII was immobilised on the surface of a glassy carbon electrode, and it competed with test sample factor VIII for the HRP-labelled anti-factor VIII antibody. Peroxide and hydroquinone (HQ), were then added and the product, benzoquinone (BQ), was reduced electrochemically. The resulting current was measured.

The electrode probe was used only once for each sample reading and so required polishing, between each immobilisation. Heut *et al.* (1993), described a method for regenerating the electrode surface electrochemically by anodisation at 2.2 V vs SCE. This eliminates the need for polishing, thereby reducing the time between sample readings.

Boitieux *et al.* (1989), devised a novel method for dissociating immunocomplexes from the electrode surface. A competitive inhibitor of β-galactosidase (β-gal) was immobilised on the electrode surface via a membrane. Catalase-labelled anti-α-fetoprotein (AFP) antibody and β-galatosidase-labelled anti-AFP antibody (different epitope) were allowed to react with the analyte (AFP) in solution, and then allowed to bind to the electrode surface (β-gal binds to its immobilised inhibitor). The production of O_2 by enzymatic reaction involving catalase was measured at a the Clark-type electrode. The sensor was easily regenerated with borate buffer, pH 10, which dissociates the inhibitor - β-gal complex in 2 minutes, ready for the next immunoassay.

Electrochemical detection of an immunoassay product does not always occur at the electrode surface. Some authors employed electrochemical techniques to measure the analyte after eluting from an immunoreactor. Creatine kinase (MB isoenzyme) was removed from serum by passing it through a bead column of immobilised antibody. The analyte was then eluted from the column, injected into the electrochemical cell and a product of its enzymatic reaction (NADH), detected electrochemically at +0.36 V (Toyoda *et al.*, 1985). The sensor could determined CK-MB concentrations in the range 20-250 U/L. Yu *et al.* (1994), performed a heterogenous sandwich ELISA for thyroid stimulating hormone, on antibody-immobilised beads, and detected the product of the enzyme reaction, electrochemically. Alkaline phosphatase catalysed the conversion of p-aminophenyl phosphate to p-aminophenyl, which was injected into the electrochemical cell and detected at +325 mV using a glassy carbon electrode (vs Ag/AgCl).

All the amperometric immunosensors described so far cannot be considered true sensors, in that following analyte addition to the system, further user-mediated steps need to be

performed before signal readout. The following homogenous assays eliminate the requirement for such additional steps. A homogenous enzyme multiplied immunoassay technique (EMIT) for the drug theophylline was adapted by Athey *et al.* (1993), to produce a rapid amperometric immunoassay, requiring only 50 µl of whole blood. Glucose-6-phosphate dehydrogenase (G6PDH)-labelled theophylline and theophylline (in blood sample) competed for anti-theophylline antibody (in assay buffer). On binding with the antibody, the activity of the enzyme is blocked, and therefore, the activity of the unbound enzyme is directly proportional to the concentration of theophylline in the blood. NADH, generated by the enzymatic reaction was detected between 12-16 minutes after the reaction was initiated, at +150 mV (vs Ag/AgCl), using platinised activated carbon electrodes. A novel homogenous assay was developed which required a photochemical cell to detect electrochemically (anodic oxidation) generated luminescence. Luminol-labelled antibodies were mixed with the antigen in solution, followed by addition of hydrogen peroxide. The luminescence of luminol is greatly decreased on antibody binding to its antigen, due to steric hindrance of electrochemiluminescence. The unbound luminol was oxidised above +0.4 V (vs Ag/AgCl), and the resulting luminescence detected optically (Tanaka *et al.*, 1993). The signal was inversely proportional to antigen concentration.

A electrochemical DNA sensor for gene detection is being studied by Hasimoto *et al.* (1994). The DNA intercalator, daunomycin, is electroactive when oxidised at a low potential, 446 mV, and gives a high current density of 6.5 µA/cm^2. The intercalator binds to double stranded DNA (hybridised), but not single stranded DNA (unhybridised), and so can electrochemically distinguish between the two. The sensor may find applications for detecting genes with implications in pathological disorders.

1.4.1.2 Potentiometric affinity-based biosensors

Potentiometric biosensors operate on the principle of measuring the charge density accumulation, at the sensing electrode, brought about by some selective process (such as an enzyme reaction), compared with a reference electrode, under conditions of zero current flow (Hall, 1990). The sensors obey the Nernst equation relating electrical potential to the log of the concentration of a known species in solution;

$$E=E^0+2.303\left(\frac{RT}{nF}\right)\log a_i$$

where E^0 = standard cell potential

R = gas constant

T = temperature

n = charge on the primary ion

F = Faraday constant

a_i = activity of the primary target ion

By far the most common type of this kind of transducer are ion-selective or gas-selective enzyme electrodes. Ion-selective membranes, composed of metal salts or polymer membranes containing ion-exchangers or neutral carriers, are fixed on the sensing electrode (Solsky, 1990). The enzyme is immobilised on, or is physically retained close to the selectively permeable membrane. The conversion of a substrate to a product by the enzymatic reaction, changes the activity of an ion or gas for which the membrane is selective. Gas-selective electrodes represent a development on ion-selective electrodes. The gas itself is not measured directly but via a change in some other parameter, such as pH, due to the production of the gas, e.g. CO_2. The majority of cell and tissue-based sensors are based on ion-selective electrodes (Solsky, 1990).

Affinity-based potentiometric sensors are generally based on labelled systems, such as enzyme-linked immunosorbent assays. Fonong and Rechnitz (1984), reported a homogenous immunosensor method which utilises a CO_2 gas-sensing membrane electrode. Human IgG (antigen) was mixed with chloroperoxidase (CI-POD)-labelled anti-human IgG. CI-POD catalyses the bromination of β-ketoadipic acid, causing the release of CO_2. On binding to the antibody, the antigen blocks the activity of the enzyme. The gas was detected by the CO_2-selective electrode, and the concentration of the gas produced is inversely proportional to the antigen concentration. The sensitivity of the sensor lay in the μg/ml range.

A new approach to the construction of potentiometric immunosensors was taken by Ghindilis *et al.* (1992). Their immunosensor for insulin determination employed the enzyme label, laccase. Laccase is able to catalyse the electroreduction of oxygen by transferring electrons directly from a carbon-based electrode surface to the oxygen molecule via the active site of the enzyme as follows;

$$O_2 + 4H^+ + 4e^- \longrightarrow 2H_2O$$

An antigen inhibition competitive assay for insulin was set up by immobilising insulin to the electrode surface. The electrode was composed of the water insoluble polymer polyethylenimine. This material was suitable because it binds carbon black and prevents non-specific adsorption of protein. Sample insulin competed with the immobilised insulin for laccase-labelled anti-insulin antibody. On binding to the immobilised insulin, in a manner inversely proportional to insulin concentration, the labelled antibody is close enough to the electrode surface to catalyse the reduction of oxygen. This results in a rapid increase in the electrode potential, the rate of this increase being indirectly proportional to the concentration of insulin in solution. No reaction substrates need to be added to this system since the substrate reagents involved are atmospheric oxygen and electrons.

Catalytic antibodies are antibodies that catalyse chemical reactions and do so by providing a microenvironment in which the reaction can proceed (the antigen-combining site), which lowers the potential energy of the transition state for the reaction. A novel potentiometric affinity sensor employing catalytic antibodies as the molecular recognition element was described by Blackburn *et al.* (1990). The catalytic antibody was raised against the transition state analogue of the hydrolysis reaction of phenyl acetate to acidic products. The resulting decrease in pH was detected by an underlying potentiometric pH electrode. The advantage of using catalytic antibodies is that the antigen rapidly dissociates from the active site of the antibody and so the sensor is regenerable.

Initial attempts at the direct, label free, potentiometric detection of analyte binding to an affinity surface were not very successful, due to interferences by salt ions and non-specific binding. Antibodies and some antigens are proteins, and in aqueous solution proteins have a net electrical charge, the polarity and magnitude of which depends on the isoelectric point and on the ionic composition of the solution. When an antibody and antigen bind the net electrical charge of the complex will be different than the electrical charge of the individual proteins. Janata (1975), tested this theory using Concanavalin A (Con A), a molecule which binds strongly to the carbohydrate, yeast mannan. Con A was covalently attached to the surface of a thin dense poly(vinylchloride) membrane deposited on a platinum wire. When the Con A 'immunoelectrode' was measured against a Con A electrode with blocked binding sites, there was a dependence of steady state potential difference on the concentration of yeast mannan. The signal produced however was extremely small. Similar experiments with human chorionic gonadotropin

(hCG) and anti-hCG were carried out by Yamamoto *et al.* (1980). The sensor, however, did not work with real samples (urine) as it was subject to interference from urine components.

Keating *et al.* (1984), attempted to amplify the response of direct antibody-antigen binding in a potentiometric ionophore-modulation immunoassay (PIMIA). Ionophores are neutral carrier ligands which complex metal ions, e.g. the antibiotic valinomycin is selective for K^+ ions. The principle of the PIMIA described was as follows. Ionophore-labelled digoxin was incorporated into a plastic support membrane and this membrane was mounted in the sensing tip of a conventional potentiometric membrane electrode. The electrode was exposed to a constant activity of marker ion (K^+), which bound to the ionophore. Charge separation occurred at the interface due to the selectivity of the ionophore, to produce a stable and reproducible background current. When anti-digoxin antibodies were added to the background electrolyte, a potential change proportional to the antibody concentration was produced. The addition of anti-digoxin antibodies always produces a potential change in the direction of increasing apparent potassium ion activity. The direct binding sensor could be used with a competitive immunoassay format.

1.4.2 Solid state transducers

1.4.2.1 Field effect transistors

Field effect transistors (FETs) operate on the same principle as potentiometric sensors, measuring charge accumulation. Ion-selective field effect transistors are essentially insulated gate FETs, in which the usual gate metal electrode has been replaced by a suitable selective membrane and a reference electrode (Figure 1.4).

A semiconductor is characterised by the limited amount of the free charge carriers. The total concentration of free carriers can be increased by impurity doping. Taking a silicon semiconductor, if there is an excess of positive mobile charges, the silicon is p-type. If the silicon has an excess of negative mobile charges (electrons), the silicon is n-type. The mobile free charged carriers are affected by electric fields, making it possible to control the concentration and behaviour of these charge carriers by external means. A FET is composed of a p-type (positive) silicon substrate into which two n-type (negative) regions, the source and the drain have been embedded.

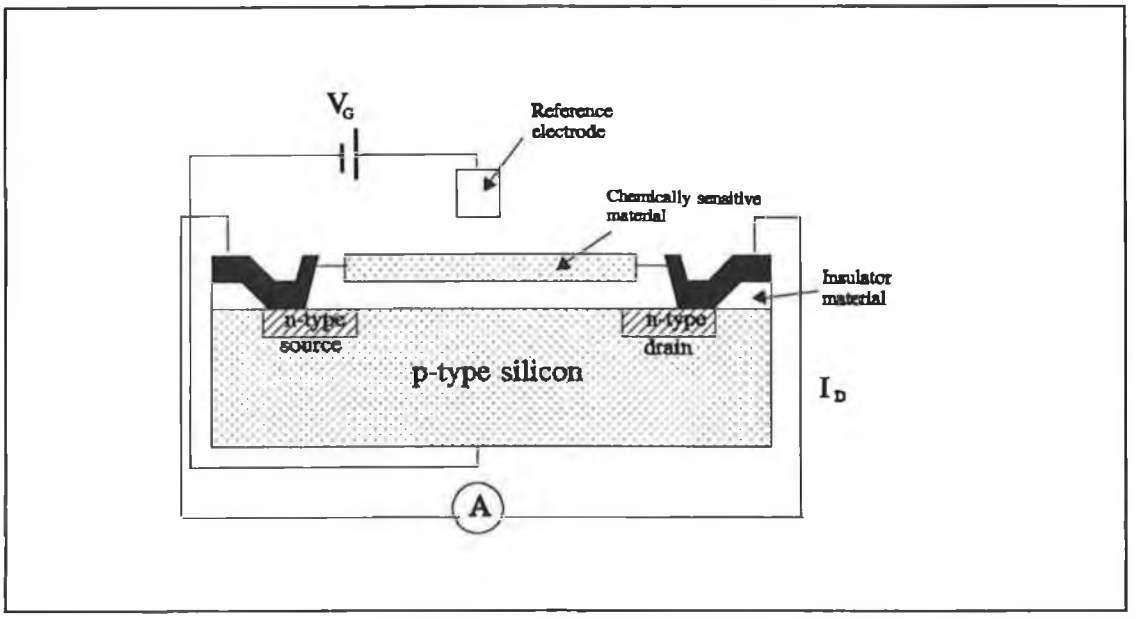


Figure 1.4: Ion-selective Field Effect Transistor

In a normal FET, when a voltage is applied between the gate electrode and the p-type silicon substrate, electrons are attracted or repelled on the surface of the substrate under the gate. When this voltage is of a certain magnitude, the p-type substrate undergoes inversion to n-type. This results in a conducting channel developing between the drain and the source n-type regions, and a drain current flows. This drain current is dependent on the electrical resistance of the inversion layer, which in turn depends on the concentration of the ionic species present.

For biosensors based on ISFETs, an ion-selective membrane (e.g. H^+-, Na^+- or K^+-selective) is placed over the gate insulator of the FET and the biologically active species, is immobilised on this. The sensor is then exposed to the analyte solution and any strongly binding species or catalytic reaction which alters the charge density at the gate changes the drain current. Development of FETs which can detect antibody-antigen interactions directly by direct reading of interfacial charge density changes have been mostly unsuccessful. Poor charge variation associated with antibody-antigen interactions, non-specific responses due to interfering ions and the requirement for very high charge transfer resistance to maintain the solution-membrane interface polarised (Hall, 1990), have attributed to this failure. Consequently, most affinity-based FET systems involve enzyme-labelled assays in conjunction with ISFETs.

A IMMUNOFET device was described by Colapicchioni *et al.* (1991), employing glucose oxidase-labelled antibody assays. The antibody was immobilised onto the ISFET gate surface via a protein A membrane. Two-site sandwich (human IgG) and competitive

(herbicide atrazine) immunoassays were performed, and the H^+ ions produced during the enzymatic reaction detected on the ion-selective electrode.

The IMMUNOFET described above requires that the antibody be immobilised via a membrane to the FET surface. This creates problems in terms of the reusability of the sensor. A novel ELISA for the determination of PCR (polymerase chain reaction) products was devised by Tsuruta *et al.* (1994). The system did not require any immobilisation to the FET. The primers of the PCR reaction were labelled with the haptens, digoxigenin and biotin, resulting in double stranded PCR products (DNA), with the hapten labels at both ends. The immunoassay was performed on the inside of a pipette tip, which was coated with anti-digoxigenin antibody, and required only 1µl of PCR product. When the PCR products were bound to the antibody, avidin-labelled urease was introduced, which bound to the biotinylated DNA. The activity of the enzyme was determined by dipping the pipette tip, into the pH measuring cell of a pH-FET, containing urea solution. The system was found to be extremely sensitive, in that 10 attomoles of labelled DNA in 1 µl of sample was detectable.

Gotoh *et al.* (1987), described an acetylcholine sensor composed of an ISFET and an acetylcholine receptor. The acetylcholine receptor protein is often employed in biosensing devices for the determination of cholinergic neurotransmitters. In its isolated form, the AChR is capable of identifying and binding to the neurotransmitters purely on the basis of structure. However, if the receptor is reconstituted in a lipid bilayer, the physiological activity of the receptor protein is retained and the binding event results in an influx of ions. The gate insulator was composed of silicon nitride, which is very sensitive to many different ions and surface charges. The system was composed of two modified ISFETs, one containing the acetylcholine receptor and the other containing a blank membrane. When acetylcholine was injected over the ISFET with the immobilised isolated receptor, the differential gate output voltage, shifted to the positive side due to the positive charge of acetylcholine. The initial rate of this shift in potential was found to be linearly proportional to the logarithmic value of the acetylcholine concentration, in the range 0.1-10.0 µM. When the receptor protein was immobilised in a lipid membrane, the response was amplified 3-fold, due to the additional flux of positive sodium ions towards the ISFET gate surface, as a result of the physiological activity of the receptor.

1.4.2.2 The light-addressable potentiometric sensor

The light-addressable potentiometric sensor (LAPS) is based on FET technology, and forms the basis of the microphysiometer (its manufactured version is called the Cytosensor) developed by Molecular Devices Corp., California. The theory behind LAPS is detailed in Mc Connell *et al.* (1992). Essentially, the microphysiometer is an extremely sensitive pH meter (61mV per pH unit at 37°C), exploiting the H^+ sensitivity of silicon nitride. The basis of operation is that an appropriate reverse bias potential is applied to a silicon plate, which has its surface nitride layer exposed to the solution (analyte matrix). A voltage gradient is formed immediately below the insulator. This voltage gradient causes the separation of hole-electron pairs, which have been formed by the absorption of light from the LED (Figure 1.5). An AC-coupled photocurrent results and flows in an external circuit. The amplitude of this photocurrent is a function of the solution pH.

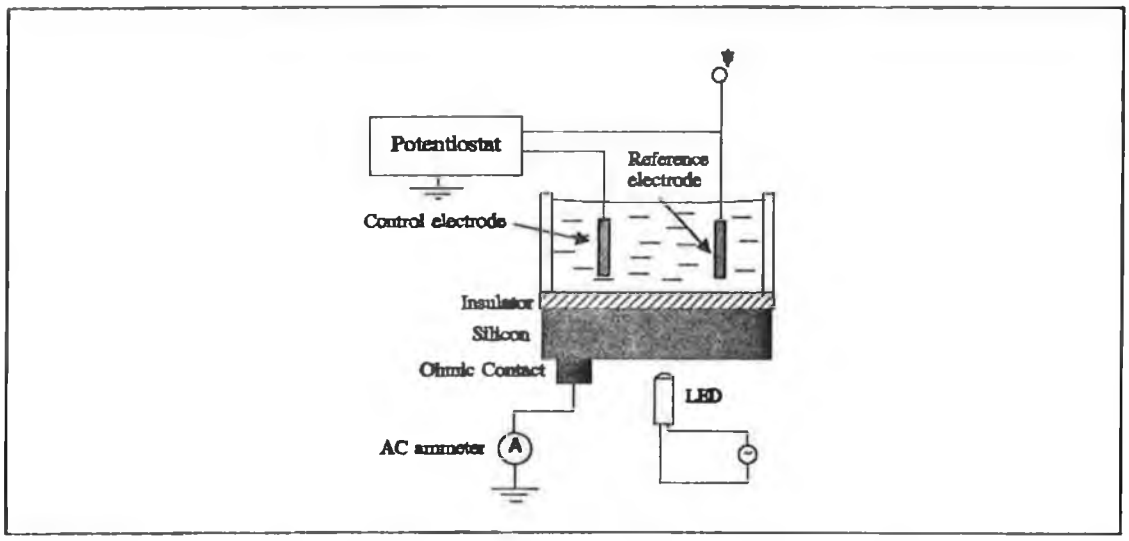


Figure 1.5: Schematic diagram of a light-addressable potentiometric sensor (Mc Connell *et al.*, 1992)

Living cells acidify their environments due to the acidic products of energy metabolism. The Cytosensor allows observation of functional ligand-receptor interactions by the monitoring of cellular metabolic rates, which is transduced as a decrease in pH.The type of response observed, both in terms of magnitude and rate, is dependent on a variety of factors such as cell type, agonist/antagonist concentration and on which physiological systems are activated. The cytosensor system was used to study the specificity of 5

muscarinic agonists for 4 different muscarinic receptor subtypes (M_1-M_4), which had been transfected separately into a Chinese Hamster Ovary (CHO) cell line (Davis *et al.*, 1993). The results obtained showed good agreement with traditional methods (phosphoinositol (PI) turnover and cAMP inhibition). In effect, the cytosensor allows bioassays to be performed, as a rapid single assay, even when the ligand-receptor interaction is coupled to second messenger pathways.

Colston *et al.* (1993), use an ELISA method for the detection of subnanogram quantities of the Mojave toxin (MoTX) with light addressable potentiometric detection. Biotin-labelled and fluorescein-labelled anti-MoTX antibodies formed a two-site sandwich immunocomplex with MoTX in solution, at 37°C for 45 minutes. The immunocomplexes were filtered onto the surface of a test-stick (streptavidin-labelled nitrocellulose paper), to which they bound via avidin-biotin interactions, and then reacted with urease-labelled anti-fluorescein antibodies, resulting in five-layer immuno-sandwich as shown in Figure 1.6.

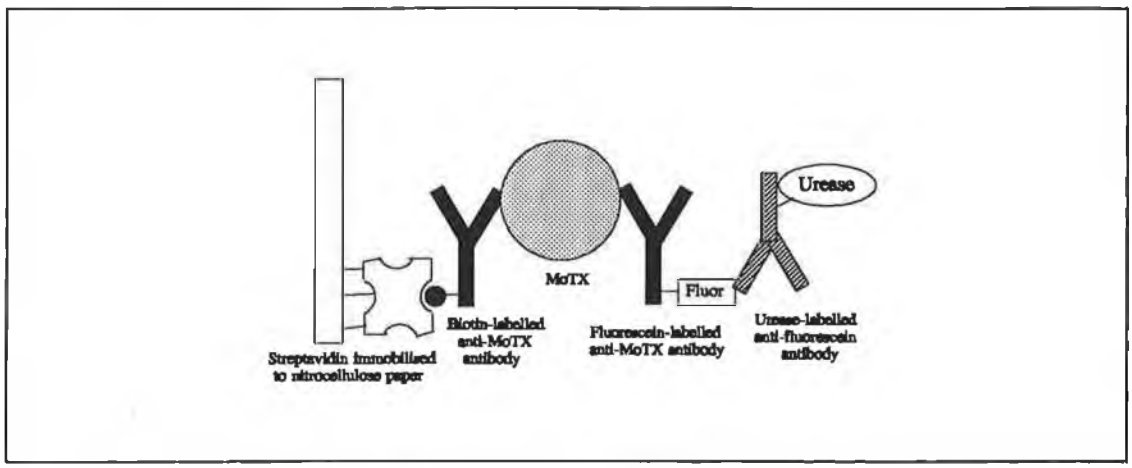


Figure 1.6: Five-layer sandwich immunocomplex for the quantitation of the Mojave toxin by LAPS (Robinett and Herber, 1994)

Each test-stick was inserted in the cytosensor and immunoconjugates were monitored via the hydrolysis of urea. A similar method for the quantitation of nanogram quantities of the recombinant tick anti-coagulant protein, was described by Robinett and Herber (1994).

1.4.3 Optical Detection

1.4.3.1 Fibre-optical systems

Biological interactions are often accompanied by changes in certain optical properties, which form the basis of optoelectronic affinity biosensors. Changes in light absorption/ fluorescence/phosphorescence/bioluminescence in the bioaffinity layer, changes in the layer thickness or refractive index, reflective behaviour of the incident light and light scattering, are used to generate measurable optical signals related to biomolecule concentration.

Optical sensor systems based on glass fibre technology, offer a high potential for biotechnological analysis. Transduction via optical fibre techniques offer many distinct advantages over other methods of transduction, some of which are outlined in Table 1.2.

	Property	Comments
Advantages	no electrical interference / absence of electrical connectors	safer to use in certain environments e.g. in the presence of explosive vapours
	no reference signal required	simple design
	thin, long, flexible and conducive to miniaturisation	suitable for use *in vivo* and in remote sensing (low loss fibres)
	inexpensive	production of disposable sensors
	can carry several wavelengths simultaneously	multi-analyte analysis
	signal can be monitored dynamically	real-time monitoring
Disadvantages	interference from ambient light	need to create a dark environment
	sample solution turbidity	interference due to scattering
	often reporter labels are required to transduce bioreactions	added complexity
	long term stability of reporter labels	photobleaching

Table 1.2: Advantages and disadvantages of optical fibre-based biosensing techniques.

Optical fibre affinity biosensors can be categorised into two major classes, *extrinsic* and *intrinsic* sensors (Figure 1.7). An extrinsic fibre sensor uses the optical fibre purely as an optical signal carrier. Light from a suitable source is transmitted into the fibre and directed to the region where it interacts with the analyte directly or an intermediate chemical/ biochemical transducer element. This interaction results in the modulation of the light intensity. The modulated light is collected and transmitted by the same or different optical fibre to the detection system, carrying with it some chemical information.

An intrinsic optical fibre sensor, on the other hand, is one in which the light is perturbed within the fibre by the parameter to be measured, and so the fibre itself is an integral part of the sensing device. Evanescent wave biosensors are an example of intrinsic sensors.

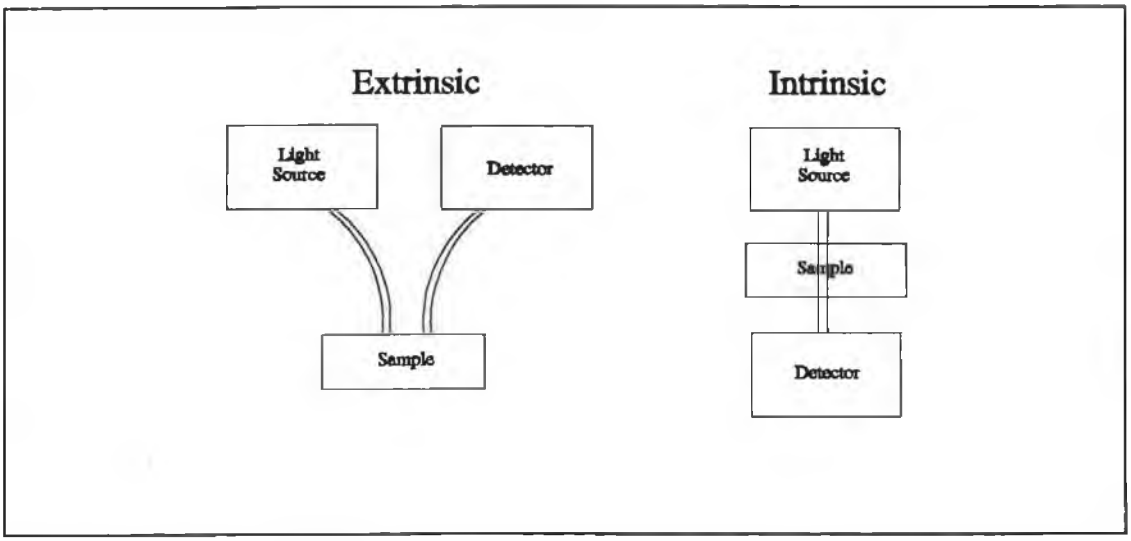


Figure 1.7: Schematic representation of extrinsic and intrinsic optical fibre sensors

1.4.3.1.1 Extrinsic fibre-optic affinity based biosensors

Optical fibres can be employed for extrinsic affinity-based biosensing, conveying light from the source to the sampling area, and from the sampling area to the light detection system. Alaire *et al.* (1990), described a regenerable immunochemical-based fibre optic sensor for the detection of the naturally highly fluorescent metabolite (BPT) of benzo[a]pyrene (BP). Its significance is derived from the fact that it is a bioindicator, found in urine, of an individuals exposure to the carcinogen BP. Monoclonal anti-BPT antibodies are retained behind a highly permeable, low cut-off membrane, at the distal tip of the fibre. The principle of operation is based on the diffusion of BPT, into the fibres reagent chamber, where it complexes with the antibody, thereby concentrating the BPT in the fibres viewing region. Readings could be made under equilibrium and non-equilibrium conditions. The sensor was not reversible due to the high affinity antibody used for sensitive analysis, but could be regenerated by flushing out the old immunoreagents and injecting fresh immunoreagents into the sensor chamber.

Most immunoassays performed at the distal end of optical fibres, required the used of an indicator molecule or label, because the analytes themselves do not have optical

properties which can be transduced effectively. Immunoassays can be performed via heterogenous or homogenous and competitive or non-competitive assay formats. A heterogenous competitive fibre optic immunosensor was described by Thromberg *et al.* (1987). A competitive equilibrium was established between labelled and unlabelled anti-rabbit IgG for rabbit IgG immobilised on the distal end of an optical fibre. The sensing region was washed following the incubation and prior to measurement, and so measurements were not in real-time. Such sensors, however, require additional steps after analyte addition and prior to signal readout, and so cannot be considered 'true' sensors. The homogenous systems described below, appear to fulfil the criteria better. Bright *et al.* (1990), employed homogenous immunoassays in a biosensor for human serum albumin. Fluorescently-labelled anti-human serum albumin, was immobilised covalently to the distal end of a fibre optic probe. On binding of the antigen (HSA), the environment surrounding the fluorescent label (dansyl chloride), is altered as the analyte shields it from the solvent water. This results in an increased fluorescence of the fluorophore. This homogenous sensor allows real-time monitoring without the addition of extra reagents. The sensor could be regenerated 50 times before there was a significant decrease in the activity of the probe. However, photobleaching of the fluorescent label was a problem.

Most antibody-based biosensors are irreversible because the affinity of the antibody must be high to ensure adequate sensitivity for most immunoassays. Astles and Miller (1993), described a sensor using antibodies with a high dissociation rate. The analyte phenytoin dissociated from the antibody with sufficient rapidity to permit rapid re-equilibration in response to a change in concentration. The antibody properties required for chemically reversible biosensor applications are discussed in Miller and Anderson (1989). The distal end sensor employed the membrane encapsulated design, and the fluorescence energy transfer between B phycoerythrin-phenytoin and Texas Red-labelled anti-phenytoin antibody. These high molecular weight conjugates are retained behind the membrane. On immunospecific binding, the fluorescence dyes come into close contact and non-radiative energy transfer (i.e. non-fluorescent), takes place from the B phycoerythrin (BPE) to Texas Red, thereby quenching the BPE fluorescence. When the low molecular weight analyte diffuses across the membrane into the reaction chamber, it competitively displaces the BPE-phenytoin, resulting in an increase in fluorescence, which is directly proportional to the concentration of phenytoin.

A novel extrinsic fibre optic immunosensor was described by Walt *et al.* (1993), which employed control release polymers and the homogenous energy transfer system, as described earlier. Fluorescein-labelled antibody and Texas Red-labelled antigen were incorporated separately into the controlled-release polymer, ethylene vinyl acetate (EVAc) copolymer, in the form of microparticle beads. These polymers release the labelled antibody or antigen slowly on contact with water. The microparticles were shown to maintain a sustained release of immunoreagents over 1000 hours. A mixture of the antibody and antigen beads were immobilised in a thin layer of hydrogel, at the distal tip of the optical fibre (Figure 1.8). The analyte diffuses into the porous hydrogel and a competitive equilibrium is established involving homogenous energy transfer. The sensor is considered reversible since the immunoreactants are continuously replenished.

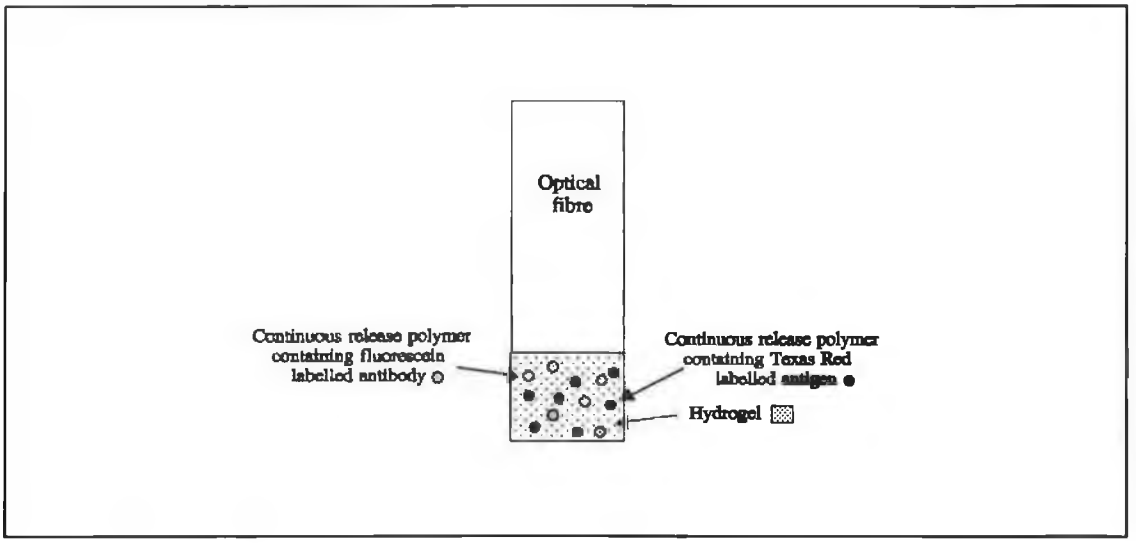


Figure 1.8: Configuration of immunosensor based on controlled-release polymer microparticles containing labelled antibody and antigen (Walt *et al.*, 1993)

1.4.3.1.2 Intrinsic fibre-optic affinity-based sensors

Intrinsic fibre optical sensors, as stated earlier, employ the fibre cable itself as an integral part of the sensing mechanism. Biosensors based on the evanescent wave fall into this class of sensors. An electromagnetic wave, called the evanescent wave, is generated at the interface between two dielectric media, when a light ray strikes the interface from the optically denser medium, at an angle greater than the critical angle. This electromagnetic radiation penetrates a short distance (of the order of a fraction of a wavelength) from the denser into the rarer medium, and decays exponentially with distance from the interface. The evanescent wave is discussed in more detail in chapter 4.

These properties of the evanescent wave, make it ideal for affinity-based biosensing. When the plastic cladding of an optical fibre is removed, the exposed core of the fibre can be placed in sample solution. This immersed probe functions as a dielectric waveguide, with the aqueous buffer as the 'cladding'. The evanescent wave is able to interact with any molecules within its penetration depth. Fluorescently-labelled immunoassays performed on the surface of the fibre, can be monitored in real-time, without the requirement for a separation step (Anderson *et al.*, 1994). The fluorescence resulting from interaction with the evanescent wave is coupled back into the same fibre and guided to the detection optics. Distal-face and evanescent wave generation of fluorescence was compared by Weber and Schultz (1992). They showed, using fluorescein solutions, that at a launching numerical aperture of 0.6, the evanescent wave signal from 2-3 cm of decladded fibre equalled the efficiency of end-face generation of fluorescence. Since the fluorescence signal is directly proportional to probe length, evanescent wave generation should be more efficient using longer probes. The intensity of proximal over distal face collection of evanescent wave generated fluorescence was investigated by Yoshida *et al.* (1988). The normalised intensities of the fluorescence signal (signal/background) for both configurations was equal, however, the collections optics required, for the proximal-end detection, are much simpler. This is because the background signal for the proximal-face collection is only due to back-reflected and scattered light, whereas the intensity of the laser light must be removed for distal-face collection.

Oroszlan *et al.* (1993), developed an evanescent wave fibre optic immunosensor (EWIS) for the determination of the pesticide, atrazine. Anti-atrazine antibodies were immobilised onto the exposed fibre core and FITC-labelled and unlabelled atrazine

allowed to compete for the limited number of binding sites. The immunosensor was sensitive within the range 0.5-200 nM. With samples of surface water and soil extracts, showing a strong luminescence in bulk emission measurements originating from the sample matrix, practically no interfering background luminescence was observed, using the evanescent wave technique. An alternative competitive fluoroimmunoassay was employed by Bier *et al.* (1992), for the evanescent wave determination of pesticides. In this case, the antigen pesticide, terbutryn, was immobilised to the exposed fibre surface. Test samples containing the pesticide competed with the immobilised pesticide for FITC-labelled anti-terbutryn antibodies. The more pesticide in solution, the less labelled antibody that could bind to the immobilised pesticide and so again an inverse relationship resulted. They claimed a detection limit of 0.1 ng/ml.

Another competition-based fluoroimmunosensor was reported by Kumar *et al.* (1994). The fibre-optic sensor quantitated the protein neurotoxin, botulinum toxin (BoTX). The sensing assay was carried out in two steps, however, exploiting a second biological affinity-based interaction between biotin and streptavidin. Biotinylated and non-biotinylated (i.e. test sample) BoTX, was allowed to competitively bind to the exposed core immobilised antibody. Following a wash-step, FITC-labelled streptavidin was passed over the fibre, which binds to the biotinylated antigen, and the fluorescence signal determined after 2 minutes. The sensitivity of the immunoassay may be increased by the biotin-streptavidin system, however, the requirement for a wash step defeats the purpose of evanescent wave immunosensing, which allows direct sensing.

Starodub *et al.* (1993), described a EWIS which could detect two drugs, lidocaine and phenytoin, simultaneously. A mixture of antibodies against both drugs were covalently immobilised on the fibre probe. Competitive immunoassays between lidocaine and B-phycoerythrin-labelled lidocaine and phenytoin and FITC-labelled phenytoin were carried out, simultaneously, on the fibre surface. Both fluorescent labels could be excited by the same laser source. The resulting fluorescence was coupled back into the same fibre, guided to a higher effective beamsplitter, and split into two, so that a set of rays fall on different detection optics. The fluorophores FITC and BPE have different Stoke's shift values (30 and 85 nm, respectively), which facilitates detection. With further development, such an immunosensor could be used for multi-analyte analysis.

Two-site sandwich immunoassays can be performed in one- or two-step formats and detected via the fibre-optical evanescent wave technique. One-step assay formats fulfil the criteria for direct signal readout after addition of the analyte. Antigen molecules with

two distinct (non-interfering) and spatially separated antibody binding sites on their surfaces can easily be employed in a one-step assay format, where one of the antibodies is immobilised and the other is labelled with the fluorophore. The labelled antibody is premixed with the antigen and then passed over the fibre, in one-step. Walczak *et al.* (1992), developed such an immunosensor for the clinically important isoenzyme, creatine kinase MB (CK-MB). The presence of elevated CK-MB, in the serum is an important indicator of myocardial infarction. Monoclonal anti-human CK-MB was immobilised to the fibre core. B-phycoerythrin (BPE)-labelled anti-CK-MB antibody, specific for a different epitope on the surface of the CK-MB, was premixed with the patient serum sample, before being passed over the fibre probe. The BPE-anti-CK-MB/CK-MB immunocomplex bound to the immobilised anti-CK-MB in one-step, at a rate directly proportional to the CK-MB concentration. The group performed some experiments to show that all background fluorescence was of surface origin and they corrected all their sample readings to take this into account. The immunoreaction was monitored in real-time and showed pseudo-first order reaction kinetics for sample concentrations of 0.1-50.0 ng/ml, during the 15 minute incubation time.

Shriver-Lake *et al.* (1993), described an EWIS for the determination of the toxin, pseudexin, and the toxin produced by *Clostridium botulinum*. They compared the sensitivity of the one- and two-step assay formats. Pseudexin has a relatively low molecular weight (16,500 Da.), and the one-step assay employed two monoclonal antibodies against two non-interfering epitopes. The two-step assay for the larger molecule, botulinum (150,000 Da.), was performed by incubating the sample with the immobilised affinity-purified polyclonal anti-botulinum antibody, followed by tetramethyl rhodamine-5-isothiocyanate (TRITC)-labelled anti-botulinum antibody. This labelled antibody was prepared from the same polyclonal antibody batch as the immobilised antibody. Since the assay is performed in two steps and the molecule is large, epitopes will still be available for the labelled antibodies to bind after the immobilised antibody has bound. They showed that the sensitivity of both assays was equivalent (1 ng/ml). However, the magnitude of the response was much greater for the one-step assay employing the monoclonal antibodies. The reason for this was postulated to be due to a combination of two factors. Firstly, the monoclonal antibodies bind non-interfering epitopes and so may allow more antigen to bind to the capture antibodies, whereas the number of available epitopes of the botulinum toxin may be reduced due to the use of polyclonal antibodies. Secondly, the pseudexin molecule has a smaller

molecular weight, and so in the immunoassay, the fluorophore may be closer to the fibre core. Since the evanescent wave penetration depth decreases exponentially with distance from the core, the further away fluorophores are from the fibre core, the lower the signal. It is noteworthy that this group tapered the optical fibres, to reduce the signal loss due to V-number mismatch (Anderson *et al.*, 1994). The fibres are partially clad and when the fluorescent signal enters the cladded portion of the fibre, a loss in signal results. This is due to the fact that the unclad portion of the fibre can support more light-carrying modes, and so some of the signal will be lost on entering the cladded portion which cannot support these modes. To compound the problem, fluorescence couples back into the highest order modes available, and so the loss of signal is elevated. Tapering the decladded portion of the fibre was shown to improve signal acquisition, the theory of which is discussed in Anderson *et al.* (1993). Feldman *et al.* (1995), determined the kinetic response and absolute sensitivity of their tapered immunoprobe by evanescent wave techniques.

Evanescent wave fibre probes have also been developed for receptor-based systems. Rogers *et al.* (1991), studied the pharmacological specificity of the nicotinic acetylcholine receptor, nAChR. Three neurotoxic peptides (α-Naja toxin, α-bungarotoxin and α-conotoxin) were labelled with FITC and the association and dissociation kinetics of the peptides for the nAChR immobilised non-covalently to the exposed core fibre surface was determined from an analysis of the time course of binding. Various agonists and antagonists of nAChR quenched the optical signal generated by FITC-α-Naja toxin binding. The inhibition constants for some of these compounds were determined from competition studies. The optical biosensor was shown to exhibit similar pharmacological profiles as those observed for membrane bound receptor.

DNA can also be used as the biological element of such evanescent wave sensors. Short oligonucleotide sequences bound to the fibre surface were used to detect FITC-labelled complementary oligonucleotide sequences (Graham *et al.*, 1992). The hybridisation was carried out at 65°C, the temperature being controlled using a jacketed flow cell. The hybridisation was reversed by raising the temperature to 80°C, and after 10-15 min, reducing it again to 65°C, ready for the next hybridisation. The rate of signal increase was linearly proportional to the oligonucleotide concentration in the nanomolar range (1-10 nM). The sensor could be used to monitor the products of polymerase chain reactions (PCR), using fluorophore-labelled primers. Gene probe biosensor assays are orders of magnitude faster than conventional filter-membrane gene probe assays.

1.4.3.2 Non-fibre based evanescent wave biosensors

The evanescent wave is generated at dielectric interfaces once the optical criteria are fulfilled (section 4.2). Planar waveguides are also available for evanescent wave fluoroimmunosensing. Badley *et al.* (1987), patented the fluorescence capillary-fill device (FCFD). The FCFD consists of two pieces of glass, separated by a narrow gap, 100 μm wide. One of the plates has a reagent layer of FITC-labelled antigen, retained within a soluble humectant coating. The other plate has a layer of immobilised antibody on its surface. When a sample is introduced into the narrow gap, the humectant layer dissolves and a competitive fluoroimmunoassay is set up (Figure 1.9). The sample volume is automatically controlled by the capillary gap. The immunoreaction is monitored by an evanescent wave generated at the surface of the plate, to which the antibody is immobilised. The fluorescence is coupled back into the planar waveguide and can be monitored by measuring the fluorescent light emerging at small angles to the axis of the waveguide. Such sensors can be mass-produced due to their simple geometry.

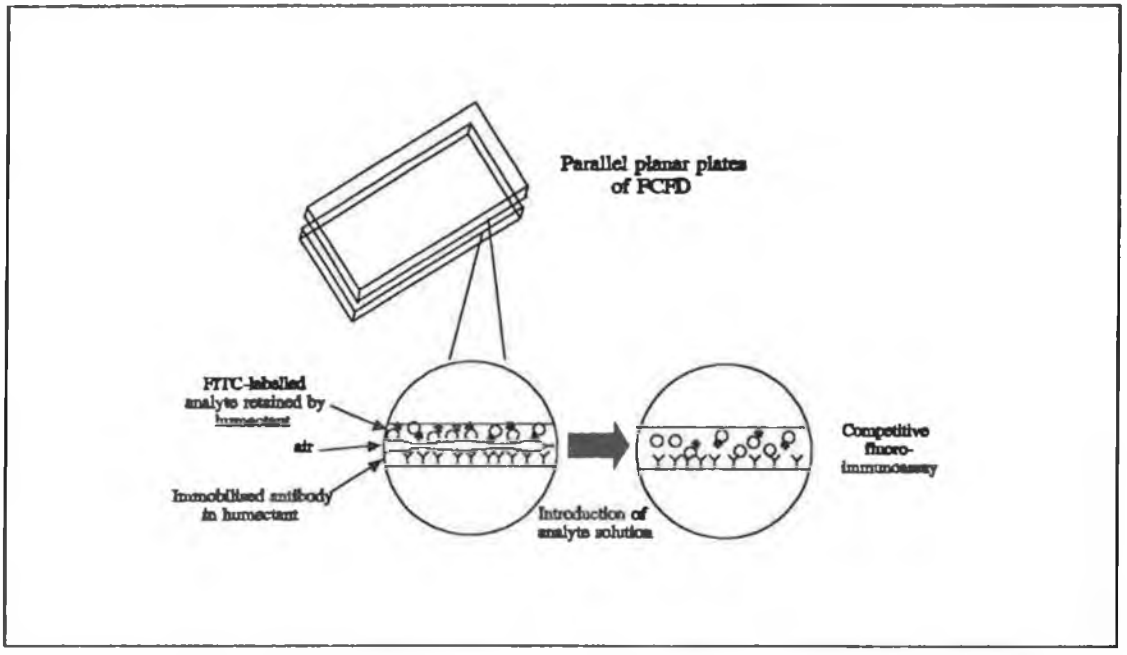


Figure 1.9: Fluorescent capillary-fill device

1.4.3.2.1 Integrated optical sensors

Input grating couplers are integrated optical sensors which can exploit the evanescent wave for label-free bioaffinity reaction monitoring. Coherent laser light is coupled into the planar waveguide by a surface relief grating. An evanescent field is generated at the sensing surface of the waveguide. The efficiency of the coupling of the laser light into the waveguide can be measured and is a direct function of the surface refractive index (probed by the evanescent wave), according to the equation (Nellen and Lukosz, 1993);

$$N = n_{air} \sin\alpha_1 + \frac{l\lambda}{\Lambda}$$

where N = effective refractive index

n_{air} = refractive index of air

l = diffraction order

λ = wavelength of light in a vacuum

Λ = grating period

α_1 = optimal angle of incidence

Hence monitoring of bioaffinity reactions at the surface, which cause a change in the refractive index can be resolved (Nellen and Lukosz, 1993). Gao *et al.* (1995), demonstrated the label-free monitoring of a sandwich immunoassay for prostate specific antigen. Changes in the effective refractive index at the surface were transduced by measuring the incoupling angle.

1.4.3.2.2 Surface plasmon resonance and the BIAcore™

Surface plasmon resonance (SPR), is an optical phenomenon arising in a thin metal film at the interface between glass and liquid, observed as a dip in the intensity of reflected light under conditions of total internal reflection in the glass.

Light shone through a prism at an angle greater than the critical angle of reflection, is totally internally reflected within the prism, and an evanescent wave is generated at the glass-liquid interface as described in section 4.2. This evanescent wave penetrates a small distance into the medium of lower refractive index, i.e. the liquid. If the interface between the two media is coated with a thin layer ($<\lambda_0$ of light) of metal, e.g. gold, and monochromatic, plane-polarised light is totally internally reflected within the prism, then the evanescent wave component of the light under certain conditions, will interact with delocalised surface electrons in the metal layer. These delocalised surface electrons are

called 'plasmons'. At a specific angle of incidence, the plasmons become excited and absorb energy, resulting in a sharp decrease in the intensity of the reflected light. The position of the dip or 'resonance angle', is sensitive to the refractive index a few hundred nanometres outside the metal film. Changes in the refractive index out to 1μm from the metal film surface can thus be followed by continuous monitoring of the resonance angle.

The best developed and most sophisticated biosensor employing surface plasmon resonance for transducing biorecognition events is the BIAcore™. The BIAcore™ (Pharmacia Biosensor AB), uses a combination of SPR and continuous flow technology to monitor interactions between molecules in 'real-time,' without the requirement for labelling. One of the biocomponents is immobilised on the surface of a gold metal film via a 100 nm thick layer of carboxymethylated dextran, which facilitates several immobilisation chemistries. This dextran matrix is mobile in three directions, and so immobilised molecules are considered to be effectively 'still in solution'. The other biocomponent is delivered in solution over the sensor chip. The BIAcore™ has an autosampler and microfluidics system to deliver all samples, buffers and regeneration solutions to the chip surface. A wedge-shaped beam of light from a light emitting diode (LED), is focused through the prism on the side of the sensor chip opposite the gold layer, and the reflected light is monitored by a fixed two dimensional array of light sensitive diodes (Figure 1.10). A detailed description of the BIAcore™ and all its components is given in Jönsson and Malmqvist (1992).

The BIAcore™ can be used to monitor any biological recognition system (e.g. antibody-antigen, receptor ligand, DNA hybridisation), if one of the biological molecules involved in the recognition event can be immobilised to the sensor chip surface. If for example, the antibody is immobilised to the sensor chip gold layer surface and the antigen is allowed to bind to it, a change in the refractive index of the solution will occur just outside the metal layer (within the sensing volume), due to the accumulation of mass at the surface. A change in the resonance angle results. All interactions are monitored continuously, as the SPR angle is directly proportional to the surface concentration of molecules. A response of 1000 response units (RU), corresponds to a concentration of about 1ng/mm^2. Some applications of the BIAcore™ and all its components are detailed in Fägerstam *et al.* (1992), and others are outlined in Table 1.3.

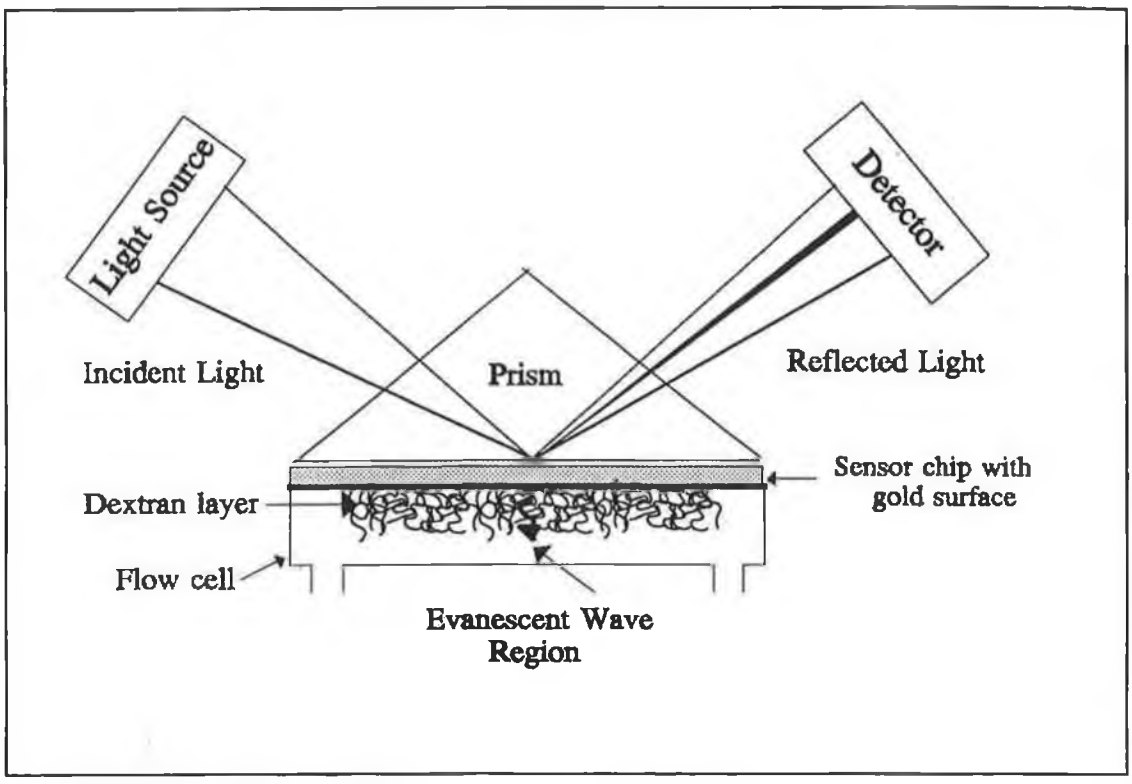


Figure 1.10: Diagrammatic representation of the BIAcore™ sensor chip surface and the associated optics for surface plasmon resonance (SPR) sensing

Biospecific Interaction	Comments	Reference
antibody-antigen	quantitation of the pesticide atrazine in drinking water by competitive immunoassay	Minunni and Mascini (1993)
receptor-ligand	exploration of the mechanism of receptor binding between the growth factor, EGF and its receptor	Zhou *et al.* (1993)
protein-DNA	verification of the hypothesis that the nuclear oncoprotein, ETS1, interacts with DNA in a sequence specific manner	Fisher *et al.* (1992)
DNA-DNA	real-time monitoring of DNA hybridisation kinetics, DNA strand separation and enzymatic modifications	Nilsson *et al.* (1995)
antibody-antigen	determination of the K_{ass} and K_{diss} of monoclonal antibodies for use in radioimmunodetection *in vivo*	Horenstein *et al.* (1993)

Table 1.3: Some applications of the BIAcore™

The BIAcore™ may find applications for the development of reversible biosensors. Antibodies with a high dissociation constant are required for reversible sensors to ensure that the sensor responds rapidly to changes in analyte concentration.

1.4.3.2.3 The resonant mirror biosensor

The resonant mirror biosensor (IAsys™), is a new biosensor launched by Fisons Applied Sensor Technology, Cambridge, UK. It is a novel design based on optical evanescent wave technology which combines the ease of use of surface plasmon resonance and the enhanced sensitivity of waveguiding techniques. It utilises a dielectric resonant structure to probe label-free affinity reactions in the sensing layer.

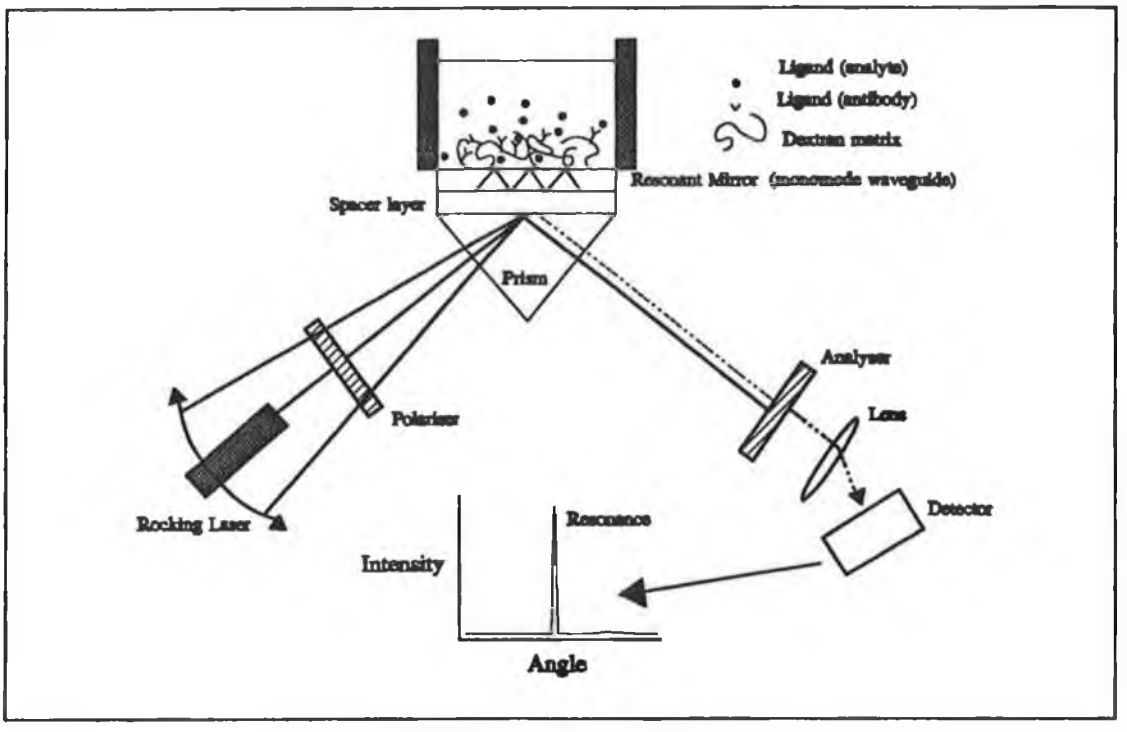


Figure 1.11: The optical and biological components of the resonant mirror-based system IAsys™ (Yeung *et al.*, 1995).

The structure of the device is shown in Figure 1.11. It consists of a glass prism, onto which a high refractive index dielectric resonant layer is deposited (100nm thick), separated by a low refractive index layer (spacer layer). Polarised light (directed at the prism by an angle which is swept repeatedly), is coupled into the resonant layer via the spacer layer, and propagates by multiple internal reflections along the monomode waveguide, before tunnelling back across the spacer layer to leave the prism. Efficient coupling only occurs for certain incident angles and for these angles an evanescent wave is generated at the waveguide surface. The evanescent wave probes the sensing layer (100 nm into the sample) and is particularly sensitive to the refractive index of the

sample. Changes in the refractive index due to bioaffinity binding at the surface, alters the resonant angle, resulting in a shift in the phase of the reflected light, which is monitored by a suitable detection system (Cush *et al.*, 1993; Yeung *et al.*, 1995). Measurement of antibody-antigen and enzyme-substrate-inhibitor interactions using the resonant mirror have been described in Buckle *et al.* (1993).

1.4.4 Piezoelectric crystal sensors

When a mechanical stress is applied to anisotropic crystals, e.g. quartz, electrical charges are induced on the surface of the crystal, due to a phenomenon known as piezoelectricity. By the converse effect, when an A.C. voltage at the resonance frequency is applied to these materials, oscillations are induced within the crystals. The optimal resonance frequency at which these oscillations are induced is highly dependent on the properties of the crystal (e.g. thickness), and also on the mass on its surface. With the addition of small amounts of bound mass to the surface, the oscillating frequency of PZ crystals, when operated in air, is decreased according to the Sauerbrey equation;

$$\Delta f = \frac{-2 f_0^2 \Delta m}{A\sqrt{\mu_q \rho_q}}$$

where f_0 is the resonance frequency of the unloaded crystal, μ_q is the shear modulus, ρ_q, the density and A, the surface area of the active crystal face. A fractional change in the frequency Δf, is equal to the fractional change caused by the added mass Δm, in the surface density of the crystal.

There are two main configurations of piezoelectric biosensor, the quartz crystal microbalance (QCM) and the surface acoustic wave (SAW) device.

1.4.4.1 Resonant quartz crystal microbalance

The QCM device consists of an AT-cut quartz crystal, sandwiched between two electrodes. An A.C.-voltage is applied causing the crystal to oscillate at its normal resonance frequency. When mass is added to the surface of the electroded crystal surface, the resonance frequency of the crystal is decreased. If bioaffinity molecules are immobilised on the surface of the crystal, the QCM device can be employed as an affinity sensor.

Ngeh-Ngwainbi *et al.* (1986), immobilised anti-parathion antibodies on a QCM, for the detection of the volatile insecticide, parathion, in the gaseous phase. Problems can arise, however, when using piezoelectric sensors in the liquid phase, in which most biological systems are found. Solvent adsorption and viscous drag can dampen the resonance frequency and cause interference (Walton *et al.*, 1991). The PZ-crystal is sensitive to variations in the density and viscosity of the active sensing layers adjacent to its surface. To overcome the problems due to liquid interactions, some researchers employed the 'dip and dry' technique, when making measurements. The resonance frequency of the biosensing crystal was determined before the crystal was dipped in the analyte solution. Following reaction with the analyte, the crystal was removed, dried (usually air-dried) and the change in resonance frequency due to the added mass determined. Herpes viruses (König and Grätzel, 1994), pesticides (Guilbault *et al.*, 1992), human granulocytes (König and Grätzel, 1993) were determined using antibody-based QCM sensors employing the 'dip and dry' technique. Fawcett *et al.* (1988) monitored the complementary hybridisation of synthetic RNAs using the same method.

The 'dip and dry' technique is rather cumbersome and time-consuming, and so research is continuing into making measurements in the liquid phase. Muramatsu *et al.* (1987), measured IgG in solution using protein A-coated crystals. The change in the resonance frequency of the crystal was measured before and after incubation with IgG (in buffered saline) by flowing deionised water over the surface, thereby eliminating the need for drying the sensor. This non-continuous method of sensing was improved on by Davis and Leary (1989), who studied the same bioreaction in liquids, using continuous measurements. They designed an oscillator circuit to compensate for the degraded quality of the AT-cut PZ crystal, in the energy absorbing medium.

Minunni *et al.* (1994), reported a PZ-immunosensor for the detection of pesticides in solution, using real-time measurements. The sensor was based on an antigen inhibition competitive assay. The same group (Skládal *et al.*, 1994), also reported the characterisation of monoclonal antibodies to the pesticide using the same sensor system. The association and dissociation kinetics were determined from real-time, frequency vs time curves.

The sensitivity of QCM sensors is often too low for certain applications. Ebersole and Ward (1988), enhanced the sensitivity of an antibody-based sensor for human chorionic gonadotropin (hCG), by performing a two-site ELISA on the surface of the crystal and developing the bound enzyme label (alkaline phosphatase), with a substrate (BCIP)

which yields an insoluble product (oxidised dimer of BCIP). The deposition of the product on the crystal surface results in an amplified decrease in resonance frequency, which is proportional to hCG concentration. This method, however, magnifies the problems of regeneration or surface cleaning between sample readings.

Another approach taken by Ghourchian *et al.* (1994), did not require the formation of a thin film on the crystal followed by fixation of the antibody or antigen. Anti-rheumatoid factor antibody was immobilised onto the surface of latex beads. The beads were added to a cell containing the PZ-crystal, followed by standard serum containing rheumatoid factor. A decrease in frequency was observed as the agglutination reaction took place, at a rate proportional to the rheumatoid factor concentration. Formation of the agglutinated latex complex, changes the density and viscosity of the solution, which in turn changes the resonance frequency of the crystal. Some non-specific binding by the antibody-bearing latex was shown to take place to the crystal surface adding to the regeneration problems.

1.4.4.2 Surface-acoustic wave devices

SAW devices can also be used for the detection of mass accumulation, by monitoring changes in acoustic wave propagation along the surface (Rayleigh waves) of a PZ-crystal. Mechanical waves, generated by an applied voltage, are propagated from a transmitter to a receiver electrode, where an output voltage is determined by an inverse piezoelectric effect. These waves are often referred to as surface acoustic waves. Anything which interacts with the launched surface wave, will alter the speed and amplitude of the wave. Bioaffinity sensors can be devised using SAW devices if one of the binding partners is immobilised on the surface of the PZ-material, in the path of the SAW. On binding with its complementary partner (e.g. antibody-antigen), the frequency of the launched wave is disrupted.

Two electronic techniques are used with SAW sensors. In one the output voltage is fed back into the input, to produce a sustained standing wave oscillation and the frequency is continuously monitored. In the other method the phase and magnitude of the output is compared with the input, and the phase difference related to the bound mass (Walton, 1991).

There are two types of SAW sensors - Lamb wave devices and shear horizontal acoustic plate mode (SH-APM) devices. SH-APM devices are preferred for liquid measurements.

SH-APM propagation does not excite any appreciable acoustic wave into the bulk solution and so is relatively insensitive to the bulk fluid properties.

Andle *et al.* (1992), described an SH-APM device and demonstrated the detection of DNA hybridisation. The biosensing device had the interdigital transducers (IDT) on the surface of the crystal opposite to where the fluid is placed, thereby eliminating any potential interactions between the biochemical solution and the IDTs. The propagation of the acoustic waves is through the bulk of the crystal and is altered by the deposition of mass. A dual delay-line device was used in which two sets of IDTs are employed (Figure 1.12). In the path of one of the oscillators, a biologically sensitive DNA coating is immobilised, while the crystal is passivated between the other set of IDTs. Systematic changes common to both delay lines, such as non-specific adsorption and temperature drift cancel out and cause little or no change in their relative response. The relative shift in frequency is measured.

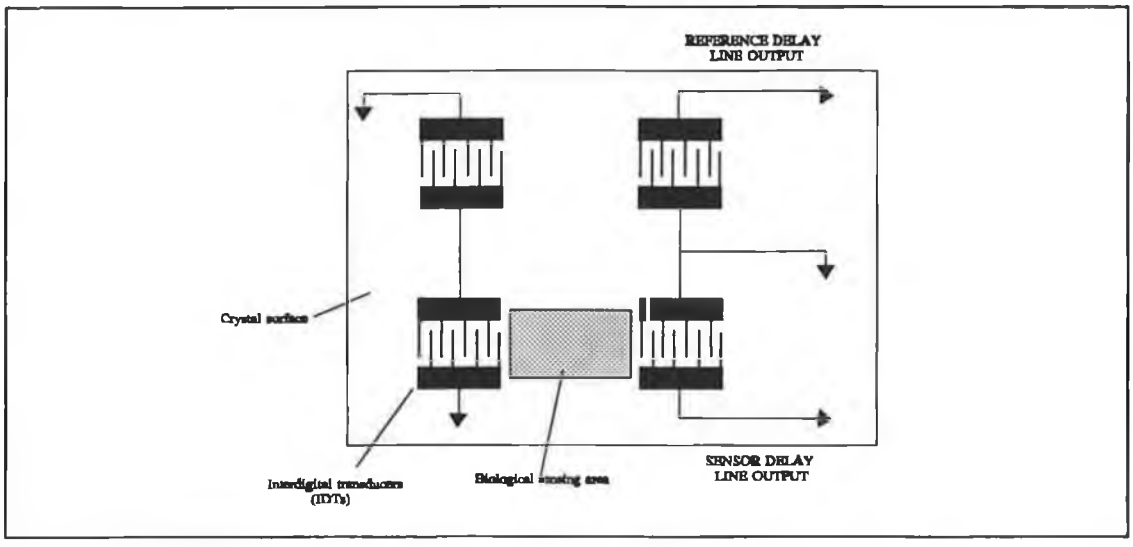


Figure 1.12: Dual-delay line configuration of a surface acoustic wave biosensing device (Andle *et al.*, 1992)

Walton *et al.* (1992), described the use of polymers with piezoelectric properties for biosensing devices. The sensitivity of SAW devices is inversely proportional to its thickness. Practically, however, there is a limit to the thinness of PZ-crystals, that can be handled. The thickness of polyvinyidine difluoride (PVdF), can be reduced greatly relative to quartz crystals. The polymer also exhibits excellent protein binding capabilities, for use in biosensors.

Aims

The aim of this project is to develop a fibre optical-based fluoroimmunosensor, which employs the evanescent wave to probe an immunoreaction occurring at the exposed fibre probe interface in real-time.

The model analyte chosen was chicken heart LDH (CHLDH), since it was readily available and inexpensive. In chapter 3, the production of specific anti-CHLDH polyclonal antibodies will be described. These antibodies will be used in the development of an enzyme-linked immunosorbent assay. The antibodies will also be labelled with the fluorescent label, FITC, for use in the development of the optical immunosensor.

The development of this optical fibre-based fluoroimmunosensor will be described in chapter 4. Different assay formats will be compared and the effect of fibre regeneration, fibre realignment and matrix viscosity on the sensor performance will be determined. The same assay formats will also be performed on the BIAcore™, a commercially available biosensor system, which also monitors immunoreactions in real-time, but in the absence of a label species. The effect of the ratio of the number of FITC molecules per antibody molecule, will also be described using both the evanescent wave fluoroimmunosensor and the BIAcore™.

In chapter 5, the development of an ELISA for human LDH (H_4 isoenzyme) will be examined, and the possibility of using this ELISA for the determination of the onset of acute myocardial infarction will be assessed.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

All buffers and reagents used in the experimental work were of analytical grade, and were purchased from Sigma Chemical Co., Poole, Dorset, England; BDH Chemicals Ltd., Poole, Dorset, England and Riedel-de Häen AG, Aktiengesellschaft, D-3016, Seelze 1, Hannover, Germany.

Chicken Heart LDH was kindly donated by Baxter Dade AG, CH-3186, Düdingen, Germany.

All antibodies except those produced 'in house' were purchased from Sigma Chemical Co., Poole, Dorset, England and Dako Ltd., 16 Manor Courtyard, Hughenden Avenue, High Wycombe, Bucks, HP13 5RE, England.

Bicinchoninic acid assay (BCA) kit and Immunopure® Immobilised Protein A gel were purchased from Pierce and Warriner (UK) Ltd., 44 Upper Northgate St., Chester, Cheshire, CH1 4EF, England.

BioRad protein assay reagent was purchased from BioRad Laboratories, BioRad House, Maylands Ave, Hemel Hempstead, Hertfordshire, HP2 7TD, UK.

Sephadex G-25 gel filtration matrix was purchased from Pharmacia Fine Chemicals AB, P.O. Box 175, S-75104 Uppsala 1, Sweden.

The HPLC column used for protein separation, Phenomenex Biosep-Sec S4000, was purchased from Phenomenex®, Melville House, Queens Avenue, Hurdfield Ind. Est., Macclesfield, Cheshire, SK10 2YF, England.

All solvents were HPLC-grade and were purchased from Lab-Scan, Unit T26, Stillorgan Ind. Park, Co. Dublin, with the exception of the anhydrous solvent toluene which was purchased from Aldrich Chemical Co. Ltd., The Old Brickyard, New Road, Gillingham, Dorset, England.

Mercaptopropyltrimethoxysilane (MTS) was purchased from Fluka Chemika AG, CH-9470, Buchs, Germany.

Pre-poured mini-electrophoresis 5-20% gradient gels were purchased from ATTO Corporation, 2-3, Hongo 7-chome, Bunkyo-ku, Tokyo 113, Japan.

Microtitre plate were purchased from Nunc Immunosorb, Denmark.

MicroFluor™ "W" Plates (microtitre plates for the fluorimeter) were purchased from Dynatech Laboratories, Inc., 14340 Sullyfield Circle, Chantill, Virginia 22021, USA.

Electrophoresis Systems™, LD Isoenzyme System was purchased from Ciba Corning Diagnostics Corporation, 490 San Antonio Road, Palo Alto, CA 94306, USA.

All BIAcore™ reagents (for immobilisation procedure) were obtained from Pharmacia Biosensor AB, S-75182, Uppsala, Sweden.

Optical fibre was purchased from CeramOptec Inc., Enfield, CT, USA

All fibre polishing solutions were purchased from Logitech, Glasgow, Scotland.

Equipment used is outlined in the relevant 'methods' section.

2.2 Methods

2.2 Antibody Production

2.2.1 Immunisation protocol for the production of rabbit anti-chicken heart LDH antibodies

A 1000 µg quantity of chicken heart lactate dehydrogenase enzyme (CHLDH), was dissolved in 600 µl 0.15M phosphate buffered saline (PBS), pH 7.4. An equal volume of Freund's Complete Adjuvant was added. This mixture was vortexed vigorously to form a stable emulsion. A 1000 µl sample was injected into a number of sites intramuscularly, into the back of a New Zealand White rabbit. The animal was boosted on day 28, using an emulsion prepared as above except that Freund's Incomplete Adjuvant was used. The animal was bled 11-14 days later from the marginal vein in the ear. The cycle was repeated to achieve a sufficiently high titre and 20 ml aliquots of serum taken as required.

2.2.2 Immunisation protocol for the production of rabbit anti-human LDH (H_4 isoenzyme) antibodies

A New Zealand White rabbit was immunised with 500 µg quantities of human lactate dehydrogenase enzyme (H_4 isoenzyme), prepared as described in section 2.2.1 . Serum was taken as required.

2.3 Preparation of serum

Whole blood taken from the marginal vein in the ear of the rabbit was allowed to clot at room temperature for 1 h. The clot was loosened away from the sides of the universal and centrifuged (Heraeus Labofuge GL) at 3000 rpm for 20 min. The supernatant was removed and centrifuged again to remove any remaining red blood cells. The resulting serum was stored frozen at -20°C in suitable aliquots.

2.4 Antibody purification

2.4.1 Ammonium sulphate salt precipitation

Rabbit antiserum was cooled to 4°C and the pH adjusted using 1N NaOH to between 7.0 and 7.4. An equal volume of cold 100% (w/v) saturated ammonium sulphate (S.A.S.) was added dropwise to the serum, stirring continuously at 4°C for 1 h. The suspension was then centrifuged at 3000 rpm for 15 min. The precipitate was washed twice with

45% (w/v) S.A.S. and, following centrifugation, the pellet was solubilised in a minimum volume of PBS. The protein solution was dialysed overnight at 4°C against 1 L PBS, with at least 2 changes of buffer.

2.4.2 Affinity chromatography

2.4.2.1 Preparation of affinity column (for purification of anti-chicken heart LDH antibodies)

A 1.4 g quantity of CNBr-activated Sepharose (Sigma) was suspended in 11 ml 0.001M HCl and transferred to a sintered glass funnel, where it was washed over 15 minutes with 280 ml 0.001M HCl followed by 8 ml coupling buffer (0.1M $NaHCO_3$ + 0.5M NaCl, pH 8.3). Chicken heart LDH (30 mg) was dissolved in 10 ml coupling buffer and added to a conical flask. The washed gel was then added. This gave a gel to buffer ratio of 1:2. The gel and antigen were mixed for 2 h with gentle shaking at room temperature. The gel was returned to the sintered glass funnel and washed with 18 ml coupling buffer. It was then transferred to a flask containing 10 ml blocking buffer (0.2M glycine in coupling buffer, pH 8.0) and left on a shaker for 1 h at room temperature.

To remove non-covalently bound protein, the gel was washed with three cycles of alternating pH, consisting of 30 ml 0.1M acetate buffer containing 0.5M NaCl, pH 4.5 and 30 ml 0.1M Tris buffer containing 0.5M NaCl, pH 8.5 . The gel was suspended in 0.02M PBS, pH 7.4, and added to a 50 ml syringe barrel. The column dimensions were 2cm high x 2.5cm diameter. A layer of PBS (0.02M) was then added to prevent the gel from drying out.

2.4.2.2 Use of affinity column

The column was washed with 30 ml 0.02M PBS, pH 7.4, to remove any protein which may have leeched from the gel during storage. Fractions (2 ml) were collected and the absorbance of these fractions monitored at 280 nm to ensure it was less than 0.03 absorbance units before applying the resuspended pellet sample (section 2.4.1). The dialysed sample (following S.A.S. precipitation) was added slowly to the column, followed by washing with 0.02M PBS to elute unbound material from the column. Fractions (2 ml) were collected and monitored for the presence of protein at 280 nm. The washing process was continued until no more protein was detected in the fractions (i.e. absorbance < 0.03).

The washing buffer was allowed to run to the top of the gel and then dissociating buffer (0.1M glycine/HCl, pH 2.5) was added. Fractions (1 ml) were collected in eppendorfs containing 100 µl 0.1M Tris/HCl, pH 9.0, which alters the pH of the eluted immunoglobulin to neutral. This quick adjustment of pH prevents denaturation of the antibody. Those fractions containing protein (checked by BCA protein assay, section 2.7.1), were pooled and dialysed overnight at 4°C against 0.1M PBS, pH 7.4 . The column was regenerated by washing with 20 ml each of 0.1M Tris/HCl, pH 8.0, 0.1M sodium acetate, pH 4.0 and 0.02M PBS, pH 7.4 . The column was stored for reuse, at 4°C in 0.02M PBS containing 0.02% (w/v) NaN_3.

2.4.3 Protein A chromatography (for purification of anti human-H_4LDH antibodies)

Five millilitres of ImmunoPure® Immobilised Protein A gel (Pierce Chem. Co.) was poured into a 10 ml syringe barrel. The column (3cm x 1.5cm) was equilibrated with 25 ml binding buffer (0.1M Tris/HCl buffer, pH 7.5). The dialysed sample (following S.A.S. precipitation), was applied to the column. Binding buffer (100 ml) was allowed to flow through the column under gravity, to remove any unbound protein. Fractions (2 ml) were then collected and monitored for the presence of protein, by measuring the absorbance at 280 nm. Once the absorbance was < 0.03, the bound protein was eluted using 0.1M glycine buffer, pH 2.5. Fractions (1 ml) were collected in eppendorfs containing 100 µl 0.1M Tris/HCl, pH 9.0, to adjust the pH back to a more physiological pH. Fractions were monitored at 280 nm for the presence of protein, and those containing protein were pooled and dialysed against 1 L PBS, at 4°C, with at least two changes of buffer.

The Protein A column was regenerated with 30 ml 0.1M citric acid, pH 3.0 and stored for reuse, in doubly distilled water containing 0.02% (w/v) NaN_3.

2.5 Enzyme-linked immunosorbent assay (ELISA)

2.5.1 Screening for antibody production

Serum from immunised animals was screened for the presence of specific antibodies, by an enzyme-linked immunosorbent assay. Each well of 10 columns of a 96-well microtitre plate (Nunc Immunosorb), was coated with 100 µl antigen (5 µg/ml), in carbonate buffer, pH 9.6 (0.01M sodium carbonate, 0.035M sodium hydrogen

carbonate). Carbonate buffer was added to the two remaining columns. The plate was incubated at 4°C, overnight. (Alternatively, the plate could be incubated at 37°C for 1 h or for 2 h at room temperature). The plate was washed five times with 0.15M PBS containing 0.05% (v/v) Tween 20 (PBS-Tween), and once in 0.15M PBS, pH 7.4 (PBS). Each well of the plate was then coated with 200 µl 3% (w/v) BSA prepared in 0.15M PBS, pH 7.4, and incubated at 37°C for 1 h. The plate was then washed once with PBS, inverted and shaken dry.

Sera to be tested, or control serum, was diluted in 0.15M PBS, pH 7.4, and 100 µl added to suitable wells. The plate was incubated at 37°C for 2 h. Control wells containing no antibody (PBS alone) were also included. The plate was washed five times with PBS-Tween and once with PBS, as described above. Commercial anti-rabbit IgG antibody labelled with horseradish peroxidase enzyme was diluted to 1:15,000 (working dilution), in 3% BSA (w/v) in PBS. This secondary antibody was added to each well (100 µl) and the plate incubated at 37°C for 30 min., followed by washing five times with PBS-Tween and once in PBS. Substrate (100 µl) for the enzyme (0.01 g o-phenylenediamine diluted in 25ml of 0.15M citrate buffer, pH 5.0, and 5 µl of 30% (v/v) H_2O_2) was added to each well and the plate incubated at room temperature until sufficient colour had developed. The absorbance of each well was read at 405 nm using a Titretek Twinreader® Plus microtitre plate reader.

2.5.2 Labelling of anti-CHLDH antibodies and anti-H_4LDH antibodies with horse radish peroxidase enzyme

Two milligrams purified IgG was dialysed overnight at 4°C, against 0.1M sodium carbonate buffer, pH 9.0. The solution volume was reduced following dialysis to approximately 500 µl by reverse osmosis on a bed of sucrose. Horse radish peroxidase (1 mg) was dissolved in 100 µl 0.1M sodium carbonate buffer (freshly prepared) in an eppendorf tube. One hundred microlitres sodium periodate (8 mM) was added and the reaction allowed to proceed at room temperature for 2 h in the dark. The immunoglobulin solution was then added to the activated peroxidase in the eppendorf and 2-3 mg Sephadex G-25 powder (Pharmacia Fine Chemicals) added rapidly. The reaction was incubated in the dark at room temperature for 3 h. The conjugate was eluted from the reaction mixture by centrifugation at 10000 rpm for 10 min. Five percent volume $NaBH_4$ (5 mg/ml in 0.0001M NaOH, freshly prepared) was added to the

eluant to stabilise the conjugate. The solution was inverted several times to ensure good mixing and then incubated for 30 min at room temperature. Ten percent volume $NaBH_4$ was then added and the solution incubated at 4°C for 1 h. The HRP-conjugated IgG and the unconjugated IgG were purified from unconjugated peroxidase by ammonium sulphate precipitation, as outlined in section 2.4.1. The final pellet was resuspended in 500 µl PBS, and dialysed overnight at 4°C against PBS. Following dialysis, an equal volume of glycerol was added and 100 µl aliquots were stored at -20°C.

2.5.3 Determination of the working dilution of HRP-anti-CHLDH antibody or HRP-anti-H_4LDH antibody conjugate

A 96-well microtitre plate (Nunc Immunosorb) was coated with 5 µg/ml of the appropriate antigen in carbonate buffer, pH 9.6, and blocked with 3% (w/v) BSA or 1% (w/v) glycine as described in section 2.5.1. (Glycine (1%) was used for HRP-anti-H_4LDH conjugates as some non-specific binding was seen with BSA.) Dilutions of the HRP-anti-CHLDH or HRP-anti-H_4LDH (as prepared in section 2.5.2) were prepared in 0.15M PBS, pH 7.4, containing 0.05% Tween (PBS-Tween), and 100 µl added to appropriate wells. PBS-Tween was added to control wells. The plate was incubated at 37°C for 1 h. Following incubation, the plate was washed five times with PBS-Tween and once with PBS. Substrate (100 µl) for the enzyme (see section 2.5.1), was added to each well and the plate incubated at room temperature for an appropriate length of time to allow colour development. The absorbance of each well was read at 405 nm using the Titertek Twinreader® Plus microtitre plate reader. The working dilution of the conjugate was determined from the dilution curve. It was taken to be the dilution value which gave an absorbance reading, of half the absorbance reading of the upper plateau of the dilution curve.

2.5.4 Two-site sandwich (ELISA) for the detection of CHLDH or H_4LDH

Each of 10 columns of a Nunc Immunosorb 96-well microtitre plate was coated with 100 µl anti-CHLDH antibodies or anti-H_4LDH antibodies depending on the antigen to be detected (5 µg/ml), in carbonate buffer, pH 9.6 . The plate was washed and blocked with 3% (w/v) BSA or 1% (w/v) glycine as described in the section 2.5.3. Antigen samples were prepared at several concentrations (comparable to LDH levels in normal serum), in 0.15M PBS, pH 7.4, and 100 µl added to appropriate wells. Reference

negative samples were also included. The plate was incubated for 1 h at 37°C. Following incubation, the plate was washed five times with PBS-Tween and once with PBS. HRP-anti-CHLDH antibody (100 µl) was added at its working dilution in PBS and incubated at 37°C for 30 min. The plate was washed five times in PBS-Tween and once in PBS, and the label detected as outlined in section 2.5.1.

2.6 Fluorescence-linked immunosorbent assays

2.6.1 Labelling of anti-CHLDH antibody with fluorescein isothiocyanate (FITC)

Affinity purified anti-chicken heart LDH antibody was dialysed overnight at 4°C against 0.1M sodium carbonate buffer, pH 9.6. The antibody solution was concentrated on a bed of PEG (average m.w. 10,000 Daltons), until the protein concentration was between 2-5 mg/ml (checked by absorbance at 280 nm). Stock fluorescein isothiocyanate solution (10 mg/ml) was prepared in DMSO. The fluorophore was added to the antibody solution in 5 µl aliquots, with quick vortexing between additions, until 50 µg FITC was added per mg of protein. The conjugation reaction was allowed to proceed at room temperature for 3 h in the dark.

The conjugated and free antibody were separated from the free FITC by gel filtration on a 15 ml Sephadex G-25 column (18cm x 1cm) in PBS, pH 7.4, containing 0.02% (w/v) NaN_3, at a flow rate of 0.5 ml/min. The first coloured fractions eluted, corresponded to the FITC-anti-CHLDH conjugate. The fluorophore/protein ratio was estimated from the formulas given below (The and Feltkamp, 1970).

$$\frac{F}{P} = \frac{2.87 \times Abs.495nm}{Abs.280nm - (0.35 \times Abs.495nm)}$$

The protein concentration was estimated by absorbance from the following formula:

$$[IgG] = \frac{Abs.280nm - (0.35 \times Abs.495nm)}{1.4}$$

The Bio-Rad protein assay (section 2.7.2) was also used to determine the protein concentration.

2.6.2 Labelling of CHLDH (antigen) with fluorescein isothiocyanate (FITC)

The antigen, CHLDH, was labelled with FITC, as outlined for the antibody, anti-CHLDH, in section 2.6.1. The protein concentration of the antigen solution was 4 mg/ml, and so 50 µg FITC per mg of protein was used for labelling.

2.6.3 Labelling of anti-CHLDH antibody with FITC at several fluorophore/protein ratios

Anti-CHLDH antibody (1.8 mg/ml), purified by protein A affinity chromatography, was labelled at 20, 50, 100, 200, 400, 800, 1600 µg FITC per mg of protein as described in section 2.6.1. The conjugates were purified by gel filtration. A control was prepared by adding 25 µl DMSO alone to the antibody solution. The fluorophore to protein ratio and protein concentrations were estimated from the formulas shown in section 2.6.1.

2.6.4 Fluoroimmunoassays

Fluoroimmunoassays were performed in the same manner as ELISA's (see sections 2.5.3 and 2.5.4), with the following changes:

1. Dynatech 96-well fluorimeter microtitre plates were used.
2. Following the final washing step, 100 µl 0.15M PBS, pH 7.4, was added to each well. The fluorescence of each well (due to FITC-labelled antibody or antigen), was determined using the well plate reader accessory of the fluorimeter (Perkin-Elmer, Model LS-50). The excitation and emission wavelengths were set at 488 nm and 520 nm, respectively, with excitation and emission slit widths set at 10 nm and 15 nm, respectively.

2.6.4.1 One-step fluorescence-linked immunosorbent assay

Dyantech fluorimeter microtitre plates were coated with anti-CHLDH antibodies and blocked with 3% (w/v) BSA, as described for the ELISA (section 2.5.4). Antigen (CHLDH) concentrations in the range 0.015 - 10.0 µg/ml, were premixed with FITC-labelled anti-CHLDH antibodies, at their working dilution (1:100), and incubated in appropriate wells (100µl), in triplicate, for 1 hour at room temperature. H_4LDH was used as the antigen in control wells. Following the incubation step, the plate was washed five times with PBS-Tween and once with PBS, and 100 µl PBS added to each well. The fluorescence intensity of each well was determined as outlined in section 2.6.3.

2.6.4.2 Two-step fluorescence-linked immunosorbent assay

Dynatech fluorimeter microtitre plates were coated and blocked, as outlined in section 2.6.3.1. Antigen (CHLDH) samples in the range 0.015 - 10.0 µg/ml, were added to appropriate wells (100µl), in triplicate, for 1 hour at room temperature. Negative controls were also included. The plate was washed five times with PBS-Tween and once with PBS. FITC-labelled anti-CHLDH (100 µl), was added to each well, at the working dilution (1:100). The plate was incubated for 1 hour at room temperature and then the plate was washed five times with PBS-Tween and once with PBS. PBS (100 µl) was added to each well and the fluorescence intensity of each well determined as outlined in section 2.6.3.

2.7 Protein assays

2.7.1 Bicinchoninic acid (BCA) protein assay

In this assay, Cu^{++} reacts with the protein under alkaline conditions, to give Cu^{+}, which in turn reacts with BCA to give a coloured product (Smith *et al.*, 1985). Two hundred microlitres of commercial working BCA reagent (Pierce Chem. Co.) was added to 10 µl test sample or standard protein solution (10-2000 µg/ml), in 96-well microtitre plate wells. BSA was typically used as the standard protein but when IgG concentrations were being measured, this protein was used as the standard. The plate was gently agitated to ensure thorough mixing and incubated at 37°C for 30 min. The absorbance of each well was read at 560 nm using a Titertek Twinreader® Plus microtitre plate reader. Protein concentrations were determined from the standard curve. Typically, BCA standard curves for protein determination gave r values of 0.999.

2.7.2 Bio-Rad protein assay

The Bio-Rad protein assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm, when binding to protein occurs (Compton and Jones, 1985). Forty microlitres commercial Bio-Rad reagent (BIO-RAD Laboratories) was added to 160 µl of test sample or standard protein solution (5-25 µg/ml), in 96-well microtitre plate wells. Typically, BSA was used as the standard protein, however if IgG was to be determined, this protein was used as the standard. The samples were diluted with 0.15M PBS, pH 7.4, so that the protein concentration was in the range 5-25 µg/ml. The contents of each

well was mixed thoroughly using a micropipette as the solutions do not mix readily. The absorbance of each well was read at 620 nm on the Titertek Twinreader® Plus microtitre plate reader, 5-60 min after sample mixing. Protein concentrations were determined from the standard curve. Typically, protein standard curves prepared with the BioRad assay had r values of 0.990 - 0.997.

2.8 Electrophoresis

2.8.1 SDS-PAGE with Coomassie Brilliant Blue staining

Polyacrylamide gel electrophoresis (PAGE), in the presence of sodium dodecyl sulphate (SDS), was carried out using commercially available 5-20% (w/v) gradient polyacrylamide gels (ATTO Corporation, Japan).

Samples (~1mg/ml) were diluted 1:1 in solubilisation buffer (0.08M Tris/HCl, pH 6.8; 2% (w/v) SDS; 10% glycerol and 0.2% (w/v) Coomassie Brilliant Blue R-250) and boiled for 2-10 minutes. Samples (20 µl) were loaded into the wells of the gel while only 5µl (as recommended by the suppliers), of the prestained molecular weight standard mixture (Sigma SDS-7B) was loaded. The gel was run in electrode buffer, pH 8.3 (0.025M Tris; 0.192M glycine and 0.1% (w/v) SDS) at 20mA per gel using the Atto vertical mini-electrophoresis system, until the blue dye reached the bottom of the gel. Gels were stained for 15 minutes in 0.5% (w/v) Coomassie Brilliant Blue R-250 in acetic acid: water: methanol (1:10:8 v/v/v) and destained overnight in the same solvent system.

2.9 High performance liquid chromatography (HPLC)

2.9.1 HPLC with UV/vis detection

Purified antibodies and antibody conjugates were prepared as outlined in the relevant sections. These preparations were characterised by HPLC. The HPLC system used was a Beckmann System Gold programmable, solvent module 126 and detection module 166. The absorbance units full scale were set at 0.015, with detection set at 280 nm. The proteins were separated by gel filtration using a Phenomenex® Biosep-Sec S4000 10 µm column. The mobile phase was 0.1M sodium phosphate buffer, pH 7.0 (filtered and degassed), at a flow rate of 0.75 ml/min. Samples (20 µl) were injected into the system at concentrations between 0.5-1.0 mg/ml and the resulting chromatograms recorded.

2.9.2 HPLC with photo-diode array detection

Antibody-fluorophore conjugates were analysed by HPLC with photodiode array (PDA) detection. The system consisted of a Waters 990 Millipore PDA detector; Waters 990 plotter; Waters 990 Millipore pump; Waters 990 30 chromatogram measure APC 3 computer and a protein Pak SW 300, 10 µm column. The mobile phase used was 0.1M sodium phosphate buffer, pH 7.0 (filtered and degassed), at a flow rate of 0.5 ml/min. Conjugate and non-conjugate samples (20 µl), were injected into the system, at protein concentrations of between 0.5-1.0 mg/ml. The peak of interest for each sample chromatogram was scanned from 230-500 nm using the PDA facility, and the resulting contour pattern recorded.

2.10 Fibre immunosensor fabrication

2.10.1 Fibre Polishing

Plastic clad silica fibre of core diameter 600 µm (CeramOptec Inc., Enfield, CT, USA), was used for the fabrication of the optical immunosensors. The fibres were first cut into ~11 cm lengths using a fibre cutter. Both ends of these fibres had then to be polished to give an optical finish.

Approximately, 40 of these fibres were placed in a polishing chuck and fixed firmly in place using cementing wax. The fibre rods were left standing proud of the polishing chuck by approximately 1 cm. Three chucks were set up in the chuck-holder, as shown in Figure 2.1, and a spirit level was used to ensure that the fibre ends were parallel to the lower face of the chuck and chuck-holder, and in exact line with one another. The chuck-holder containing all of these rods was then placed on a Logitech PM2A polishing rig (Logitech Ltd., Glasgow, Scotland), as shown in Figure 2.2.

The polishing rig has 2 polishing plates, a steel plate for use with 9µm and 3µm polishing suspensions and a polyurethane plate for use with 0.125 µm polishing suspensions. A semi-circular mechanical arm sweeps across the radius of the polishing plate. The chuck-holder is placed in the semi-circular arm and as the arm sweeps across the rotating polishing plate, a spinning action is induced into the chuck holder. This spinning action improves the quality and uniformity of the polished surface. The rig also has a rotating drum mechanism which deposits polishing suspensions, dropwise, onto the rotating plate. The 9 µm Al_2O_3 suspension was used first, until the majority of the cracks were removed from the fibre end face. The grinding action of this fluid,

combined with the rotation of the steel polishing plate, for a duration of approximately 3 h, polishes the end faces of the rods. The polishing solution was then replaced with the 3 μm Al_2O_3 fluid and the process continued. The final polishing was carried out using the polyurethane plate and the 0.125 μm polishing suspension, to give a finer optical finish. After 3 hours, the fibre end face surfaces were examined microscopically. The fibre rods were removed from the chucks once the polishing was deemed satisfactory. The other ends of the fibres were now set up in the system exactly as before and the process repeated. The result is a bundle of ~10 cm long plastic clad silica fibre rods with both end faces polished.

Following cleaning with water and then alcohol, to remove any remaining polishing suspension and cementing wax, ~8 cm of the plastic cladding (primary coating) surrounding the fibre core was removed using a sharp scalpel, taking care not to damage the silica fibre core. The silicone cladding was removed using an etchant solution based on a methylene chloride / sulphuric acid mixture, commercially available from Lumer (Bagnolet, France). The declad rods are left in contact with the etchant solution for about 15 min and then washed with water, followed by ethanol. An ethanol soaked lens tissue was used to wipe each rod individually, to ensure complete removal of cladding.

2.10.2 Antibody immobilisation to fibres

2.10.2.1 Cleaning of fibres

The exposed core was acid cleaned by immersion in a 1:1 mixture of concentrated HCl and methanol for 30 min. Following washing several times in ultra pure water, the fibres were immersed in concentrated H_2SO_4 for 30 min and washed in ultrapure water. The fibres were then placed in a beaker of boiling ultrapure water for 30 min. The fibres were then allowed to air dry.

2.10.2.2 Silanisation of fibres

A 2% (v/v) solution of mercaptopropyltrimethoxysilane (MTS) was prepared in anhydrous toluene and the acid-cleaned exposed core optical fibres were immersed in this solution for 2 h at room temperature. The reaction was carried out under an atmosphere of nitrogen. Following silanisation the fibres were rinsed in dry toluene and allowed to air dry.

2.10.2.3 Crosslinking of antibodies to the exposed core of optical fibres

N-gammamaleimidobutyryloxy succinimide ester (GMBS), was dissolved in 100 µl of dimethylformamide (DMF), and diluted in absolute ethanol to a final concentration of 2mM. The fibres were immersed in this solution for 1 h and then washed in 0.15M PBS, pH 7.4. The purified antibody solution was diluted in PBS to a final concentration of 50 µg/ml. The silanised and crosslinked exposed core optical fibres were immersed in the antibody solution for 1 h at room temperature. A ratio of 1 fibre per millilitre of solution was maintained, i.e. 20 fibres were immersed in 20 ml antibody solution. Antibody coated fibres were stored, until required, in 0.15M PBS, pH 7.4, containing 0.02% (w/v) NaN_3, as preservative.

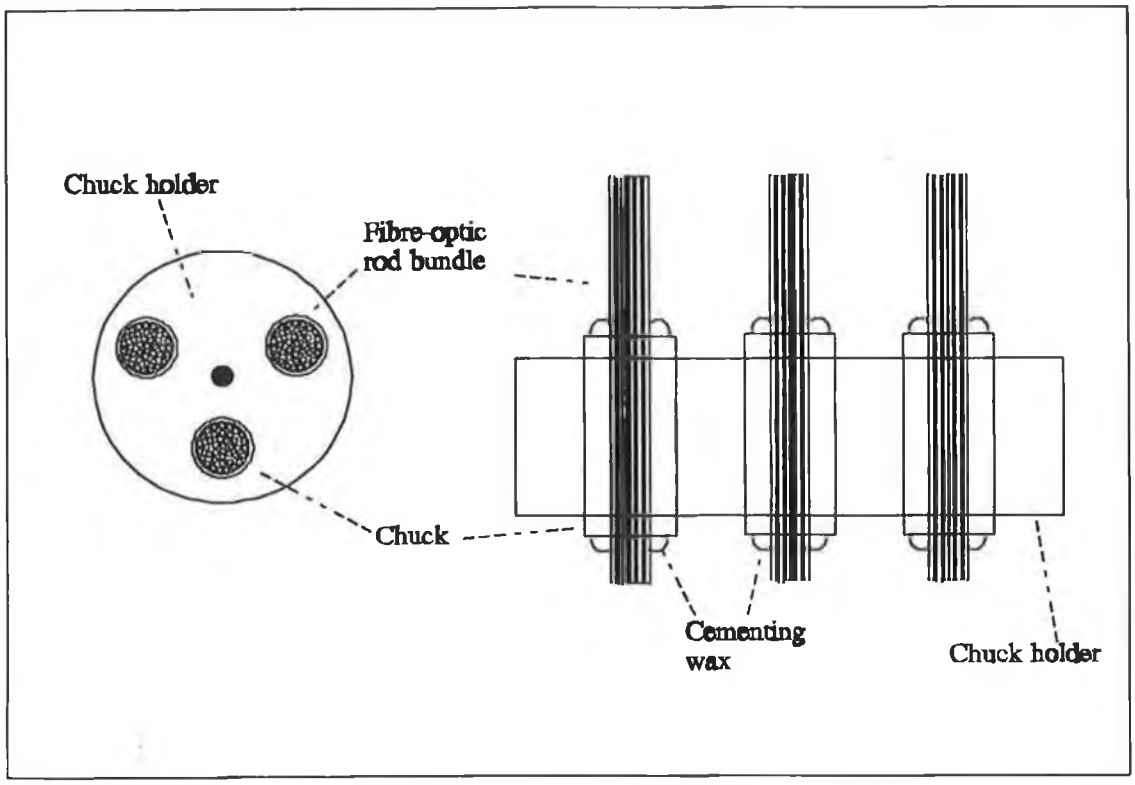


Figure 2.1: Plan and Side View of Fibre Set-Up for Polishing

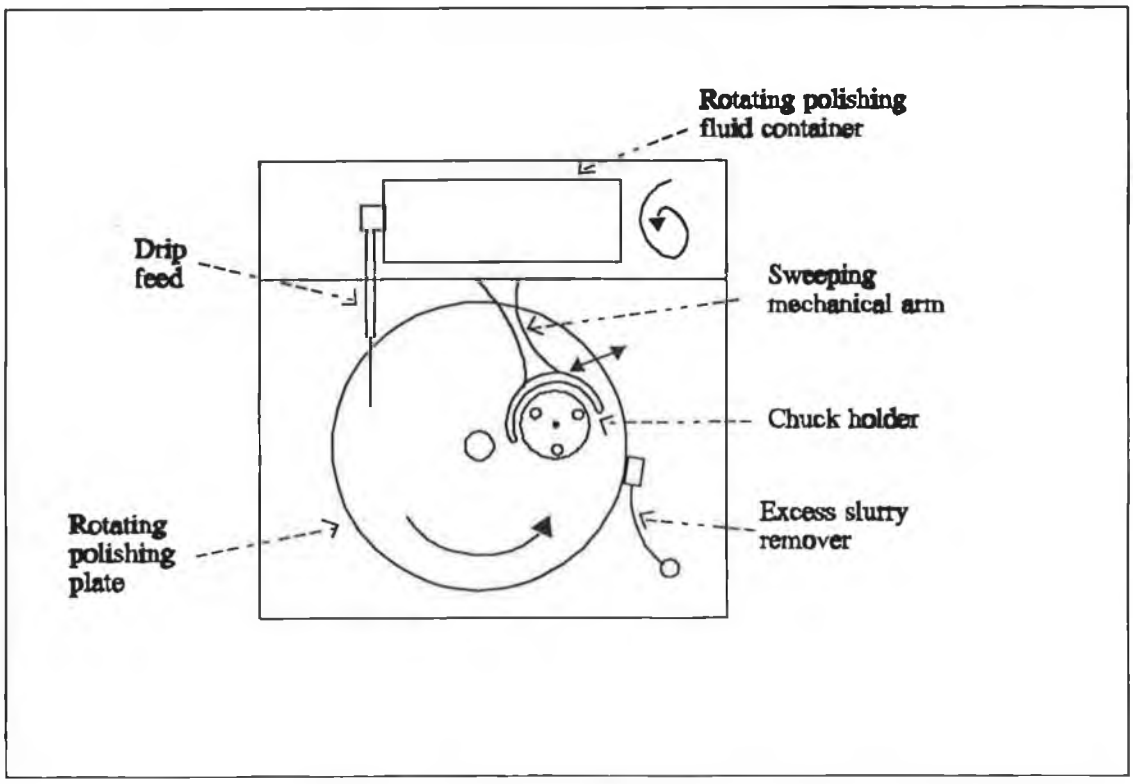


Figure 2.2: Logitech PM2A Polishing Rig

2.11 Fibre ELISAs

2.11.1 Working range of fibre probes

One-step and two-step enzyme-linked immunosorbent assays were performed on the fibre surfaces, in order to determine the working range of the fibre probes.

Fibre probes coated with affinity purified anti-CHLDH antibodies, were incubated in 1% (w/v) BSA in 0.15M PBS containing 0.05% (w/v) Tween 20, for 1 h at room temperature, to block the non-specific binding sites. For the two-step assay, each fibre was then incubated, in triplicate, in 3 ml CHLDH samples (0.39-10 µg/ml) in 1% BSA/PBS, (one fibre per test tube, such that 5.5 cm of the fibre length was covered), for 15 min at R.T. Each fibre was washed five times in 4 ml PBS-Tween and once in PBS alone. Three millilitres HRP-anti-CHLDH (working dilution, 1:4000), in 1% BSA/PBS, was then added to each test tube and incubated for 30 min at R.T. The fibres were again washed five times with PBS-Tween and once with PBS. Substrate (3 ml) for HRP (see section 2.5.1), was added to each test tube and incubated at 37°C for 30 min. The reaction was stopped by removing the fibres from the test tubes. The absorbance of each sample solution was measured at 405 nm on the Shimadzu spectrophotometer UV-160A.

A one-step assay was performed in a similar manner, except that the antigen samples and the second antibody were premixed for 1 min prior to incubation with the fibre. Controls (fibre probes coated with anti-mouse IgG) and blanks (no antigen) were also included.

2.11.2 Reproducibility and optimisation of antibody immobilisation procedures

The optimal coating concentration and the reproducibility of the immobilisation procedure was determined by performing an ELISA on the fibre surfaces. Commercially available HRP-anti-rabbit IgG (3 ml) was incubated with the rabbit anti-CHLDH coated fibres for 30 min at R.T. Following washing five times with PBS-Tween and once with PBS, the fibres were incubated with the substrate for HRP (see section 2.5.1), for 30 min at 37°C. The reaction was stopped and read at 405 nm, as described in section 2.11.1. All coating concentrations were performed in triplicate. Goat-anti mouse IgG-coated fibres were used as the controls.

2.12 Evanescent wave immunosensing

2.12.1 Optical System

The optical system used for the immunosensing experiments is outlined in Figure 2.3. In order to detect evanescent wave excited fluorescence, the system was required to separate the excitation radiation from the returning fluorescent light, as efficiently as possible.

An argon-ion air-cooled laser (Cathodeon, Oxford, U.K.), with a 488 nm line was used as the excitation source. This corresponds very closely with the excitation maximum of fluorescein. The light from the laser is passed through expanding optics; a microscope objective (A) of numerical aperture (N.A.) 0.65, and a collimating lens (B). The light is then reflected from a Raman holographic edge filter C, (Physical Optics Corporation, Torrance, CA, U.S.A.), and launched via a microscope objective (D), of N.A. 0.65, to the antibody (or antigen) coated fibre, which is mounted in the flow cell. The thicker dotted line represents the expanded and collimated laser beam light. The holographic edge filter is designed to be used off-axis from 5-20 degrees. The reflecting mirrors E and F, are adjusted such that angle α has a value of 12°. This corresponds to 0% transmission at 488 nm and approximately 77% transmission at 520 nm, the emission maximum of FITC.

The flow cell (manufactured by AGB Scientific), consists of a 9 cm long glass tube, of internal diameter 0.4 cm, with inlet and outlet ports at opposite ends and opposite sides, as shown in the diagram. Screw-on caps at both ends, hold rubber septums in place, through which the fibres are threaded and held firmly in place during the experiments. Flow of liquids through the flow cell was controlled by a peristaltic pump at 5 ml/min. The flow cell is held in position in a metal frame which is attached to an X-Y-Z micropositioner, thus allowing optimisation of the launching position of the fibre.

The evanescent field of the guided radiation excites the fluorescein within the penetration depth (due to the immunoreaction) and a fraction of the fluorescence is captured by the fibre. The returning fluorescent light is coupled back along the same path, by higher order modes. It is transmitted by the holographic edge filter (which blocks 488 nm and transmits 520 nm), and is then focused by the optical lens (G) onto the detecting optical arrangement.

The fluorescein fluorescence is detected by a Hamamatsu photomultiplier tube (PMT), whose signal is amplified using a Ithaco current sensitive amplifier (model number

1642), and fed into a lock-in amplifier, LIA (Stanford Research systems, model SR510), for synchronous detection. The chopping wheel (I), operating at a frequency of 213 Hz, provides the reference signal for the LIA. A glass filter, H (Reynard Corp., San Clemente, CA, USA), which blocks 488 nm to 0.01% and transmits 520 nm at 95% was placed in front of the PMT for further spectral filtering of the exciting radiation from the fluorescence. The detected analogue signal was converted to digital format, and then passed to the PC for storage. The computer software measures the fluorescence signal as a function of time.

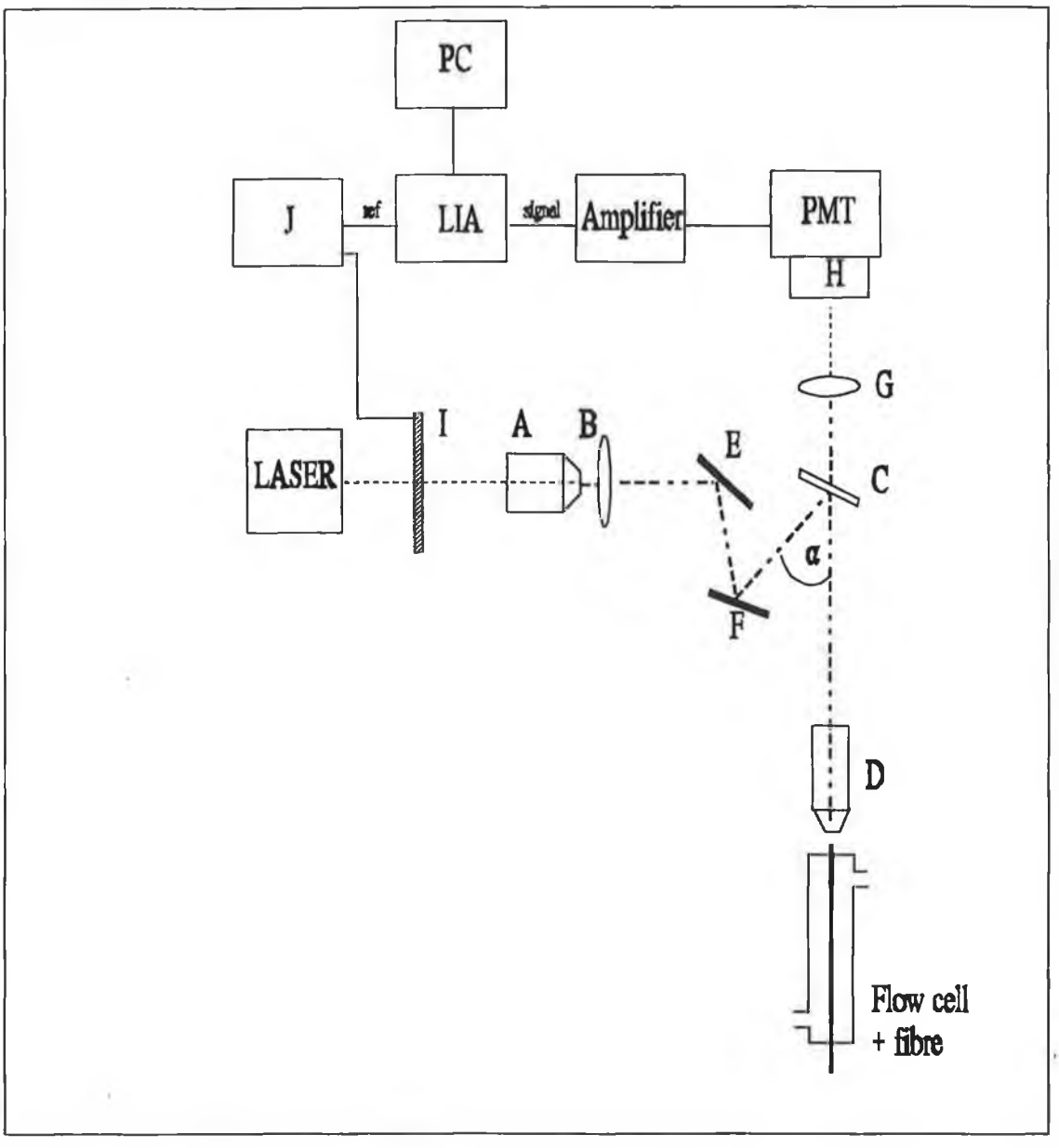


Figure 2.3: Optical Arrangement for Evanescent Wave Immunosensing

A: microscope objective
B: collimating lens
C: holographic edge filter
D: microscope objective N.A. 0.65
E,F: reflecting mirrors
G: focusing lens
H: glass filter
I: chopping wheel
J: chopping wheel controller

2.12.2 Evanescent wave immunosensing assays

Fibre probes with immobilised anti-CHLDH antibodies were incubated in 1% (w/v) BSA in 0.15M PBS, pH 7.4, until a steady baseline was established. The sample was prepared in the same solution and pumped into the flow cell at 5 ml/min, without exposing the fibre to air. Once the sample filled the flow cell, the pump was stopped. For all the immunoassays, a new fibre was used for each sample concentration. Controls (goat anti-mouse antibody coated-fibres) were included in all experiments. The fluorescence signal was monitored for approximately 4 min, and the initial rate of fluorescence increase determined.

2.12.2.1 Two-step immunoassay

For the two step assay (Figure 2.4 (1)), the fibres were preincubated with different concentrations of CHLDH (30-10000 ng/ml) in 1% (w/v) BSA/PBS, for 15 min. FITC-labelled anti-CHLDH (20 µg/ml), in 1% (w/v) BSA/PBS was pumped into the flow cell, at 5 ml/min, and the fluorescence signal recorded.

2.12.2.2 One-step immunoassay

CHLDH (39-10000 ng/ml) prepared in 1% (w/v) BSA/PBS, was premixed with 20 µg/ml FITC-anti-CHLDH and within 1 min, injected into the flow cell at 5 ml/min, for the one-step assay (Figure 2.4 (2)). The fluorescent signal was recorded.

2.12.2.3 Direct immunoassay

FITC-labelled CHLDH samples (section 2.6.2), at concentrations ranging from 125-5000 ng/ml, were pumped directly into the flow cell at 5 ml/min, for the direct assay (Figure 2.5 (1)). The fluorescent signal was recorded.

2.12.2.4 Competitive immunoassay

CHLDH (0.1-10 µg/ml) was premixed with an excess of FITC-CHLDH and pumped into the flow cell at 5 ml/min. The CHLDH and FITC-CHLDH competes for binding to the fibre-immobilised antibody (Figure 2.5 (2)). The fluorescent signal was recorded.

2.12.2.5 Regeneration experiment

A one-step assay (2000 ng/ml CHLDH + 20 µg/ml FITC-anti-CHLDH premixed) was performed 10 times, using the same fibre probe. The fibre was regenerated between experiments by pumping 0.1M glycine, pH 2.5, through the flow cell for 1 min.

2.12.2.6 Effect of increased FITC labelling on anti-CHLDH affinity using the evanescent wave immunosensor

Fibre probes coated with antigen (CHLDH) were used for this experiment. Anti-CHLDH purified by protein A affinity chromatography (section 2.4.3), was labelled at several fluorophore to protein ratios (50-1600 µg FITC per mg of protein) as outlined in section 2.6.3. The effect of increased labelling on antibody affinity was determined by pumping 100 µg/ml of each sample into the flow cell. A new fibre was used for each sample. The fluorescence signal was monitored for 7 min.

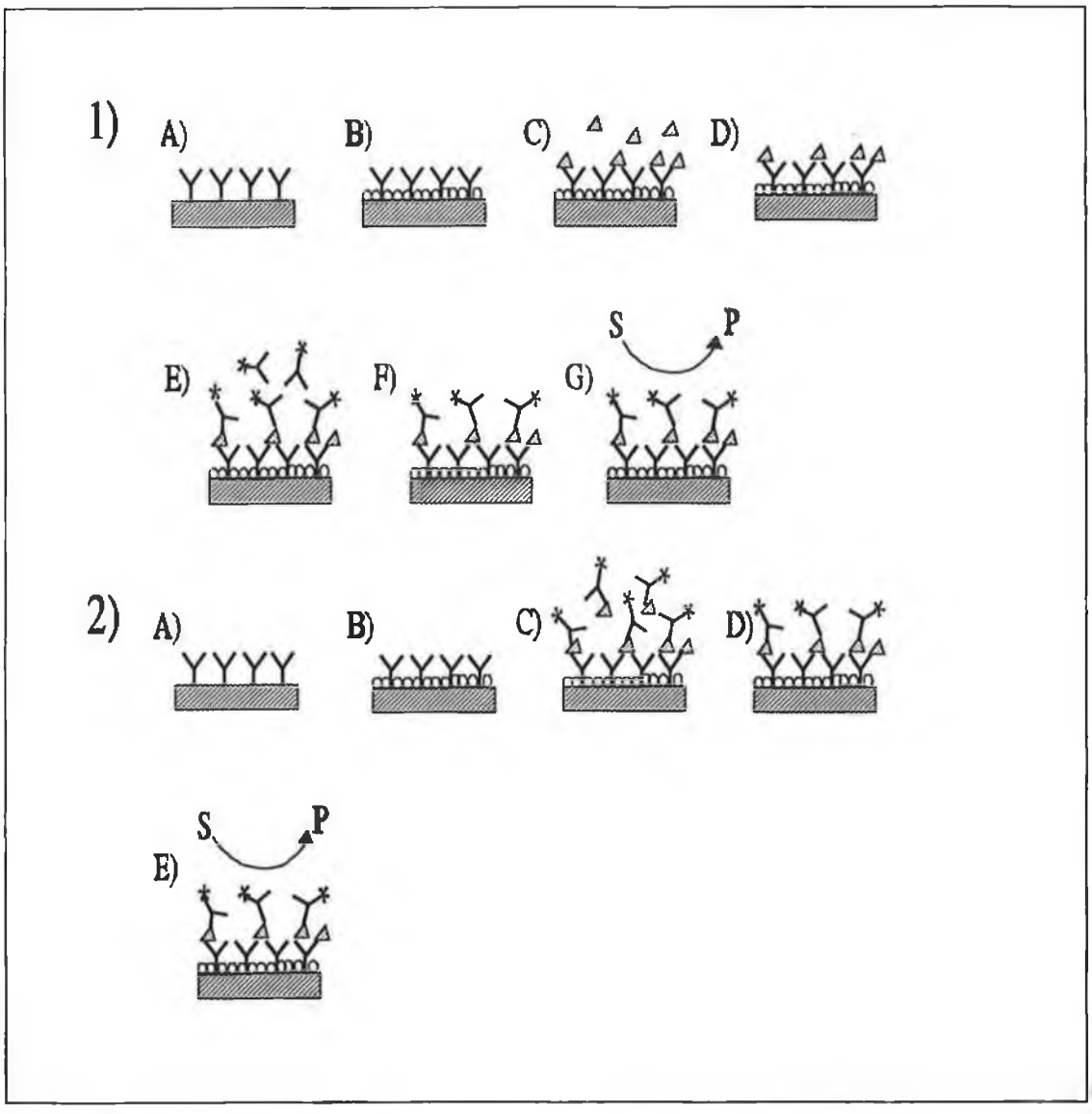


Figure 2.4: Schematic representation of enzyme-linked or fluorescence-linked immunosorbent assays. The antibody is immobilised to the solid surface (A), and the non-specific binding sites on the surface are blocked with BSA (B). In the two step assay (1), the antigen is incubated with the immobilised antibody (C), followed by a washing step to remove any unbound antigen (D). The second labelled antibody is then added (E), followed by a washing step to remove any excess labelled antibody (F). If the label is a fluorophore, it is measured fluorimetrically. If the label is an enzyme, substrate (S) for the enzyme is then added, and the product (P) measured optically (G). In a one step assay (2), the antigen and second antibody are premixed and incubated with the immobilised antibody (C). The excess antibody and antigen are removed by washing (D) and the label detected as before (E).

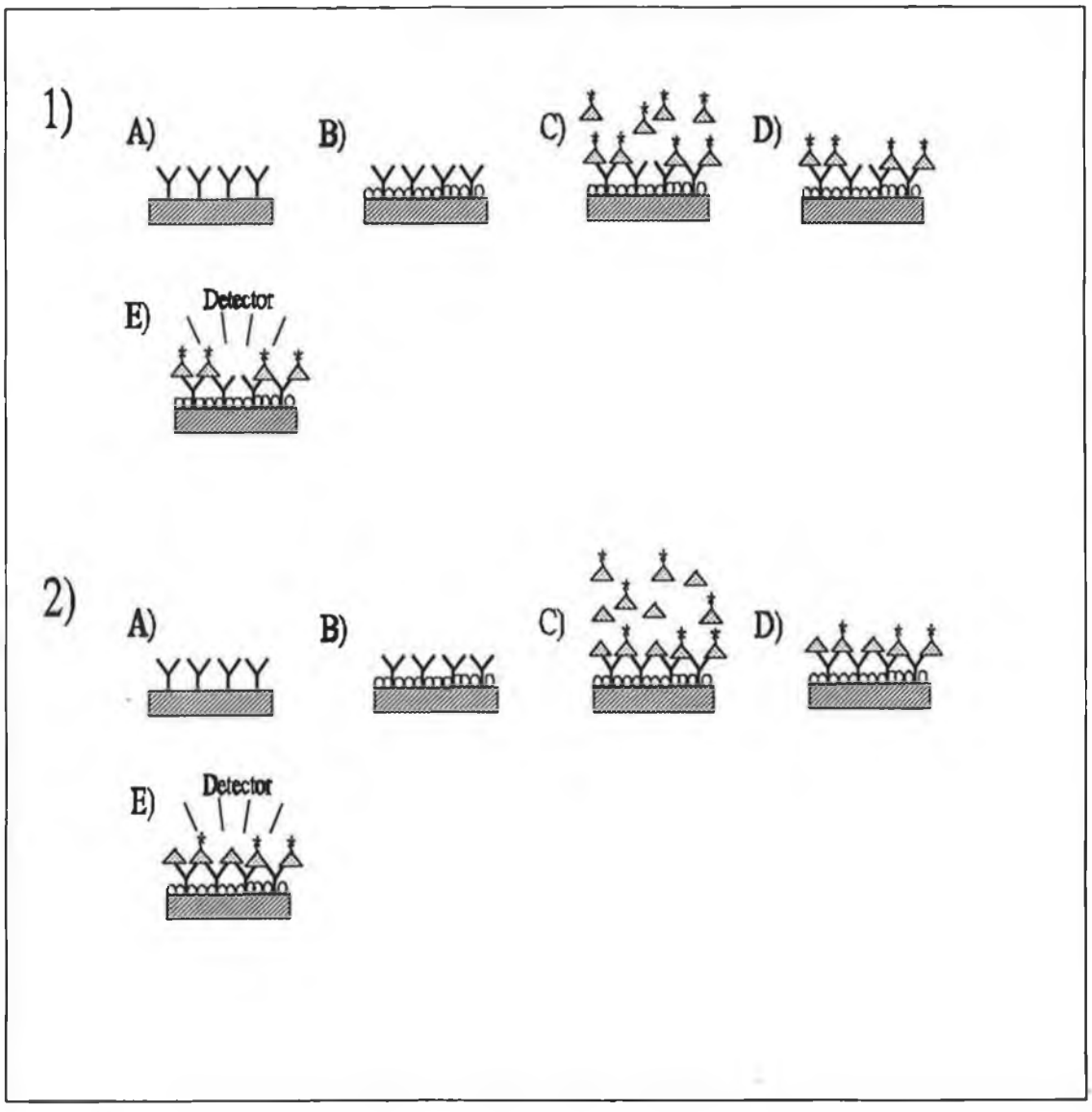


Figure 2.5: Schematic representation of direct and competitive fluorescent-linked immunoassay. The antibody is immobilised to the solid substrate (A), and the non-specific binding sites on the surface blocked with BSA (B). In the direct assay (1), the FITC-labelled antigen is incubated at various concentrations with the immobilised antibody (C). Unbound labelled antigen is removed by washing (D), and the fluorescence detected using a fluorimeter (E). In the competitive assay (2), unlabelled and FITC labelled antibody, compete for binding to the immobilised antibody (C). Unbound moieties are removed by washing (D) and the label detected as before (E). An indirect relationship of fluorescence to antigen concentration results, because the more unlabelled antigen present, the less antibody sites left available for labelled antigen binding.

2.13 BIAcore™ Studies

The BIAcore™ (Pharmacia Biosensor AB), is a biosensing device which operates on the principle of surface plasmon resonance (SPR). The details behind the principle of SPR and the operation of the Biacore™ are given in section 1.4.3.2.2. All BIAcore™ experiments were performed with the assistance of Mr. Gary Keating, School of Biological Sciences, D.C.U.

2.13.1 Immobilisation to sensor chip surface

Antibody or antigen were immobilised (for different applications), to the Sensor chip CM5 (Pharmacia Biosensor AB). The sensor chip was inserted into the BIAcore™ instrument with the dextran/gold surface side in contact with the flow cells. The immobilisation procedure was controlled via the BIAcore™ computer and microfluidics system.

Filtered and degassed Hepes buffered saline, HBS, (10mM HEPES, pH 7.4; 150mM NaCl, 3.4mM EDTA, 0.05% BIAcore™ Surfactant P20), was injected over the sensor chip, for 5 min at 5 µl/min. N-hydroxysuccinimide in H_2O (100mM) and N-ethyl-N′-(dimethylaminopropyl)carbodiimide in H_2O (400mM), were mixed 1:1 and 35 µl injected over the sensor chip at 5 µl/min, to activate the surface. Antibody or antigen solution (200 µg/ml), in 10mM sodium acetate buffer, pH 5.5, was then injected over the chip at a flowrate of 5 µl/min for 7 minutes. Excess reactive groups were deactivated by injecting 35 µl 1M ethanolamine hydrochloride, pH 8.5, at 5 µl/min. Finally, non-covalently bound material was removed via 2x1 minute pulses of 10mM HCl and 1x1 minute pulse of 5mM NaOH at 5 µl/min.

Running buffer (HBS) was allowed to flow continuously over the chip at 5 µl/min between experiments.

2.13.2 BIAcore™ immunoassays

Direct (antigen alone), two step (antigen followed by second antibody) and one step (antigen and second antibody premixed) immunoassays were performed in the flow channel coated with the anti-CHLDH antibody. The antibody coated chip was regenerated between each new sandwich using 2x1 min pulses of 10mM HCl and 1x1 min pulse of 5mM NaOH.

2.13.2.1 Direct immunoassay

For the direct assay (Figure 2.5 (1)), the sample containing CHLDH (9-5000 ng/ml), in 0.15M PBS, pH 7.4 was injected over the immobilised antibody chip for 2 min at a flowrate of 10 µl/min and the sensorgram recorded. Each sample was injected randomly and in triplicate with regeneration between each sample injection.

2.13.2.2 Two-step immunoassay

For the two-step assay (Figure 2.4(1)), the sample containing CHLDH (9-10000 ng/ml), in 0.15M PBS, pH 7.4 was injected over the immobilised antibody chip for 2 min at 10 µl/min, followed by 20 µg/ml anti-CHLDH (FITC-labelled) for a further 2 min at 10 µl/min, and the sensorgram recorded. This second antibody amplifies the signal and improves sensitivity. Each sample was injected randomly and in duplicate with regeneration of the surface between each sample.

2.13.2.3 One-step immunoassay

For the one-step assay (Figure 2.4(2)), the CHLDH sample (9-10000 ng/ml), in PBS was premixed with 20 µg/ml anti-CHLDH (FITC-labelled), and injected over the sensor chip, within 1 minute of mixing. The premixing was controlled by the computer and microfluidics system. The sample was injected for 2 min at 10 µl/min, and the sensorgram recorded. Samples were injected randomly and in duplicate, with surface regeneration between each sample.

2.13.3 FITC-labelled antibody studies

Anti-CHLDH antibodies were labelled at various fluorophore/protein ratios (section 2.6.3), and the effect of increased labelling on the antibody affinity assessed using the BIAcore™.

FITC-anti-CHLDH (100 µg/ml) in PBS, was injected over the CHLDH-coated chip, for 20 minutes at 2 µl/min and the sensorgram was recorded. The chip surface was regenerated using a 3 min pulse of 10mM HCl, 3 min pulse of 0.1M glycine, pH 2.5, and another 3 min pulse of 10mM HCl, at a flowrate of 2 µl/min.

2.14 Miniaturised assay for LDH determination

A colorimetric assay for total LDH determination was miniaturised so that it could be performed in 96-well microtitre plates. Test or standard serum (40µl) was added to appropriate wells of a 96-well microtitre plate. Buffered lactate solution (0.07M lactate; 0.3M Tris/HCl, pH 8.5; 0.2% (w/v) BSA) was added to each well (110 µl) and the plate incubated at room temperature for approximately 10 min. Colour reagent (7.5mM NAD; 4.0mM INT; 1.6mM PMS) was added to each well (35 µl) and the plate incubated at room temperature, protected from light, for exactly 10 minutes. One molar HCl (80 µl) was added to each well to stop the reaction. The absorbance of each well was read at 492 nm on the Titertek Twinreader® Plus microplate reader.

2.15 LDH isoenzyme analysis by electrophoresis with densitometry scanning

The five isoenzymes of human LDH were separated by electrophoresis using a commercially available kit, Electrophoresis Systems™, LD Isoenzyme System (Ciba Corning Diagnostics Corporation).

One microlitre of sample (<1000 U/L) was applied to the 1% agarose gel (LD Isoenzyme Gel) and electrophoresis carried out in the barbital buffer provided (43mM sodium barbital; 7mM barbital) for 35 mins in the Universal Electrophoresis cell. Following electrophoresis, the LDH isoenzymes were developed using the LD Reagent provided ((0.5M lithium L(+)-lactate; 15mM NAD; 7.3mM nitro blue tetrazolium and 0.2mM methoxyphenazine methosulphate) diluted in 0.4% bicine-2-amino-2-methyl-1,3-propanediol solution), at 37°C for 20 min, according to the manufacturers instructions. The gel was washed in deionised water, to remove excess LD reagent and dried in an oven at 65°C for 2 hours.

The developed gel was scanned using a transmittance/reflectance scanning densitometer (Hoefer Scientific Instruments GS300), and the relative percentages of the individual LDH isoenzymes determined by integration.

2.16 Western blotting

Western blotting of proteins was carried out using the method described in Towbin *et al.* (1979). Samples to be blotted were first subjected to SDS-PAGE electrophoresis using 5-20% gradient gels, under non-reducing conditions, as outlined in section 2.8.1. Following electrophoresis, the gel was soaked for 15 - 20 min in a bath containing 50

ml blotting transfer buffer (25mM Tris + 192mM glycine, pH 8.3, containing 20% (v/v) methanol). The gel was blotted to nitrocellulose paper (Electran®, BDH), using a wet blotting system (Pharmacia Midget System™), for 45 min at 30V, followed by 45 min at 60V.

The blot was then transferred to a bath containing blocking solution, 3% (w/v) BSA in TBS-Tween (10mM Tris/HCl, pH 8.0 + 150mM NaCl, containing 0.05% (v/v) Tween 20), for 1 h at room temperature, and then washed once with TBS-Tween. The blot was then transferred to a bath containing 15 ml 1:100 dilution HRP-labelled anti-H_4LDH antibody (section 2.5.2), diluted in 3% (w/v) BSA in TBS-Tween, and incubated for 2 h at room temperature, with gentle agitation. The nitrocellulose blot was washed five times with 15 - 20 ml TBS-Tween, and once with TBS.

The HRP label was developed with 3,3' diaminobenzidine tetrahydrochloride (10 mg DAB dissolved in 10 ml 50mM Tris buffer, pH 7.6 + 15 µl 3% (v/v) H_2O_2), which yields an insoluble brown product. The blots were developed at room temperature for 15 min, washed with deionised water and air dried.

CHAPTER 3

PRODUCTION, PURIFICATION AND CHARACTERISATION OF ANTIBODIES TO CHLDH AND THEIR USE IN THE DEVELOPMENT OF ANTIBODY-BASED ASSAYS

3.1 Introduction

This chapter is concerned with the production of polyclonal antibodies to chicken heart LDH (CHLDH), for use in the development of a model evanescent wave immunosensor (chapter 4). Antiserum was raised in rabbits, and the specific antibodies were purified by ammonium sulphate precipitation followed by affinity chromatography. The antibodies were labelled with horseradish peroxidase enzyme (HRP) or fluorescein isothiocyanate (FITC), and used in the development of enzyme-linked or fluorescence-linked immunosorbent assays, respectively, for the determination of CHLDH. Antibody and conjugate preparations were characterised by high performance liquid chromatography (HPLC), SDS polyacrylamide gel electrophoresis (SDS-PAGE) and spectral analysis.

3.2 Polyclonal antibodies.

Antibodies can be produced against any molecule capable of eliciting an immune response in a host animal. If an antigenic response is produced, then specific antiserum is obtained. This antiserum is polyclonal in nature, in that it contains a mixture of antibodies which will bind the antigen, but not at the same site or 'epitope', and with varying strength or 'affinity'. Production of polyclonal antibodies rather than monoclonal antibodies is often more practical (Cahill *et al.*, 1995). A monoclonal antibody preparation consists of identical antibodies which bind the same epitope on the antigen surface, with the same affinity. Production of monoclonal antibodies is much more time-consuming, relatively more expensive and requires a much higher level of expertise than polyclonal antibody production (Köhler and Milstein, 1975).

3.3 Immunisation.

Large molecular weight proteins (>10,000 Da), e.g. CHLDH (140,000 Da), tend to be much stronger immunogens than smaller molecules (haptens), such as drugs and hormones, which require crosslinking to a large carrier protein in order to produce an immune response (Erlanger, 1973). Although high molecular weight molecules generally elicit an immune response readily, adjuvants are used to maximise the response. Adjuvants consist of a number of substances, whose physical and/or biological properties when applied with immunogen affect the antibody response in various ways, but most importantly by potentiating class G immunoglobulin production, by increasing the

efficiency of antigen presentation. Typical adjuvants include aluminium salts, heat-inactivated *Bordetella pertussis*, liposomes and muramyl dipeptides. The most commonly used adjuvant was developed by Freund. Freund's Complete Adjuvant consists of a mixture of mineral oil, a suspension of heat killed *Mycobacterium tuberculosis* and the emulsifier Arlacel A. This preparation is used for the initial immunisation because the mycobacteria act as powerful stimulants for the cells of the immune system (Tijssen, 1985). Freund's Incomplete Adjuvant (no mycobacteria) is used subsequently for booster injections in subcutaneous sites because of its reduced pathogenic effects. The antigen solution is emulsified in the adjuvant to form a stable, oil-in-water emulsion and injected intradermally, subcutaneously, intramuscularly, intraperitoneally or intravenously depending on the physical nature of the injection, the species and the stage of the immunisation routine (Catty and Raykundalia, 1988).

The choice of animal is generally based on convenience. For polyclonal antibody production where large quantities of serum are required, the choice of animal is commonly between rabbits, goats, donkeys, sheep, horses and guinea pigs. Rabbits tend to be the most common choice since they are relatively inexpensive, easy to handle and maintain, and they yield relatively large amounts of serum (Burrin and Newman, 1991).

3.4 Antibody purification.

Removal of non-immunoglobulin serum proteins and non-specific immunoglobulin from antisera is important for many antibody applications. Specific antibodies can be purified from serum to different degrees using techniques such as ammonium sulphate precipitation, affinity chromatography (Yarmush *et al.*, 1992), absorption of antisera and HPLC (Carty and O'Kennedy, 1988). These techniques can be used singly or in various combinations.

Ammonium sulphate precipitation is commonly used as an initial crude preparation step. Salt precipitation works on the principle of differential solubility. The solubility of protein species differ according to their degree of hydrophobicity and increasing the amounts of salt will enhance hydrophobic interactions between the proteins and cause precipitation. Thus, we may selectively precipitate specific fractions of the dissolved protein in the sample. This purification step removes up to 50% of contaminating proteins.

If it is known which non-specific antibodies are present in the serum sample, these may be removed by absorbing the antiserum on a matrix to which these antigens have been immobilised. The advantage of absorption is that the harsh denaturing conditions required for the dissociation of the bound antibody in affinity chromatography are avoided.

Affinity chromatography isolates antigen-specific antibodies by immobilising the antigen to a gel matrix, typically CNBr-activated Sepharose. The antibody binds specifically to the antigen and subsequent to removal of all other proteins by washing with the binding buffer, the specific antibody is eluted by conditions which do not favour antibody-antigen binding, such as a pH change (Hudson and Hay, 1980).

Protein A affinity chromatography columns can be used to selectively remove antibodies of the IgG class. Protein A, isolated from *Staphylococcus aureus*, binds to the F_c portion of class G immunoglobulins. Again, the class G immunoglobulins can be eluted by changing the environmental conditions so that they are unfavourable for binding (Zola and Brooks, 1982). Antibodies can be further purified by HPLC, based on size exclusion methods where differences in molecular weights of proteins or classes of immunoglobulins is exploited.

3.5 Labelling of antibodies with HRP enzyme.

Horseradish peroxidase (HRP), β-galactosidase and alkaline phosphatase are the most commonly employed enzymes for labelling of antibodies for use in immunoassays. The properties of these enzymes which make them suitable for ELISA's include a) high turnover number, b) stable on storage, c) ease of conjugation, d) easily detectable activity and e) compatibility with immunoassay conditions.

HRP was employed for labelling of anti-CHLDH antibody. HRP is a glycoprotein and is therefore most easily conjugated to antibody proteins via the periodate method. The method of Tijssen and Kurstak (1984) was used. The carbohydrate residues on the enzyme are oxidised yielding aldehyde groups. These aldehyde groups are then allowed to react with the amino groups of the immunoglobulin molecule, forming Schiff bases. These Schiff bases are stabilised by sodium borohydride, which acts by reducing the aldehyde groups, thereby preventing hydrolysis. The labelled antibody and unlabelled IgG are purified from the free HRP by ammonium sulphate precipitation. HRP is soluble in solutions of $(NH_4)_2SO_4$ up to 70-80% saturation, and therefore, solutions of 50%

$(NH_4)_2SO_4$, can be used to selectively precipitate the labelled and unlabelled IgG. It has been reported that using this procedure 90% of the HRP is conjugated, retaining 90% of its activity.

3.6 Labelling of antibodies with fluorescein.

Fluorescence is the emission of a photon of light, from a molecule which has been placed in the excited state by the absorption of another photon. This emitted photon is of lower energy than the absorbed photon, and therefore, is of a longer wavelength (Ward and Fothergill, 1976).

Several fluorescent compounds have been used to label proteins, including amino-methyl-coumarin-acetic acid (AMCA), tetramethyl rhodamine isothiocyanate (TRITC) and highly fluorescent algal pigments called phycobiliproteins (Kronick, 1986), but by far the most commonly used fluorophore is fluorescein isothiocyanate (FITC), a functionalised derivative of fluorescein.

Fluorescein has several properties which makes it suitable for use as an antibody label. It has a high fluorescent yield (extinction coefficient and quantum yield), is available in a form that can be easily conjugated with proteins and does not change the isoelectric point of the protein on conjugation, is highly fluorescent at neutral pH and compatible with immunoassays conditions. However, it has a small Stoke's shift and is subject to photobleaching (Haughland, 1991).

The conjugation of antibodies with FITC proceeds by nucleophilic attack of the unprotonated ε-amino group of lysine on the fluorochrome, resulting in a thiourea bond. At pH 9.5, most of the lysines are unprotonated, and maximal efficiency of conjugation is achieved. Antibody and fluorophore are mixed at controlled ratios, depending on the protein concentration. Conjugation is favoured at high antibody concentrations, but the significance of competing hydrolysis increases at low antibody concentrations (<2 mg/ml). It has been shown that a fluorophore/protein ratio of 1-4 is optimal for labelled antibodies. However, quenching due to overconjugation is not a serious problem with FITC, as it is for TRITC (Goding, 1983).

3.7 Enzyme-linked and fluorescence-linked immunosorbent assay.

The development of methods for chemically coupling detectable entities such as enzymes and fluorophores to antibodies, so that they both retain their biological activity and the realisation that antibodies and most antigens could be immobilised to solid surfaces in functional form, led to the concept of enzyme-linked (reviewed by O'Kennedy, 1989), and fluorescence-linked immunosorbent assays (Smith *et al.*, 1981). In such immunoassays, the quantification of antigens or antibodies is facilitated by this enzyme or fluorophore labelling, as they allow detection of immunocomplexes formed on a solid phase. The fixed enzyme, once washed free of excess reagents, and on subsequent reaction with its substrate, yields a coloured product, which can be detected optically. The optical density of the coloured product can be related to antigen concentration. In the case of a fluorescent label, the fluorescence can be detected directly using a fluorimeter.

Immunoassays can be divided into homogenous or heterogenous assays. In a heterogenous assay, a separation step is required where the free antibody and antigen is separated from the complexed solid-phase bound antigen-antibody. The quantifiable signal from the complex does not differ from the free entity and therefore without this separation step, interference would occur. In a homogenous assay, this separation step is not required. The binding of antigen and antibody results in a change in the activity of the label and therefore this activity can be detected without the problems of interference.

Immunoassays can also be divided into competitive (Egan *et al.*, 1991) and non-competitive assays (Cousin *et al.*, 1992). The former is based on a competitive equilibrium between an excess of labelled and unlabelled antigen (or antibody), i.e. the analyte to be determined, for a limited amount of its binding protein. The more unlabelled species present, the less of the labelled species that will bind and an indirect relationship results. In a non-competitive assay, the analyte to be measured complexes with its binding protein bound to a solid phase. A second labelled antibody binds to this complex and is measured.

The two-site sandwich immunoassay presented in this chapter is a non-competitive, heterogenous assay. The same polyclonal antibody preparation was used as the immobilised capture antibody and the second labelled antibody. The antigen CHLDH

is large, having a molecular weight of 140000 Da, and so has sufficient epitopes for both antibodies in the sandwich (Shriver-Lake *et al.*, 1993).

Results

3.8 Production and purification of anti-CHLDH antibodies

3.8.1 Titre of anti-CHLDH antiserum

Antisera to chicken heart LDH (CHLDH) was obtained by immunisation of New Zealand White rabbits, with the commercially available purified protein (section 2.2.1). The antigen was prepared in Freund's adjuvant and injected intramuscularly. The highest antibody titre achieved was 1:32,000. The titre was taken to be the serum dilution which gave an absorbance reading of half the absorbance reading of the undiluted serum. Normal rabbit serum (taken before the rabbit was immunised), was also titred as a control (Figure 3.8.1).

3.8.2 Purification of anti-CHLDH antibodies

Specific anti-CHLDH antibodies were purified from the antiserum by ammonium sulphate precipitation and affinity chromatography as described in sections 2.4.1 and 2.4.2. A sepharose affinity column was prepared by linking CHLDH to CNBr-activated sepharose. Only antibodies specific for CHLDH, bound to the immobilised antigen. Unbound proteins were removed by washing the column with binding buffer and the specific antibodies were eluted by changing the pH of the elution buffer. Both IgG and IgM classes of antibody are isolated by affinity chromatography, as the purification is based on specificity alone. The titre of the purified antibody was determined as for the unpurified antiserum (Figure 3.8.1), and was found to be 1:32,000.

3.8.3 Characterisation of purified anti-CHLDH antibody by HPLC

A sample of the purified antibody and a commercially available, affinity-purified goat anti-mouse IgG antibody, were analysed and compared by HPLC, as described in section 2.9.1 (Figure 3.8.2). The retention times of the purified anti-CHLDH antibody and the commercial antibody preparation were 15.07 and 15.09 minutes, respectively. The smaller peaks found at retention times between 18-20 min, were shown to be due to the preservative thiomersal and glycine salts. The commercial preparation has an extra peak at 13.91 min, which is probably due to slight antibody aggregation during storage.

Figure 3.8.1

Production of anti-CHLDH antibodies. Serial dilutions (1:100 - 1:128000) of normal rabbit serum (◊), unpurified antiserum (■) and affinity-purified anti-CHLDH antibodies (□) were prepared in 0.15M PBS, pH 7.4 and the titre determined as outlined in section 2.5.1. The antiserum and purified antibody preparations have a titre of 1:32000, which is an indication that there is a large quantity of specific antibody present.

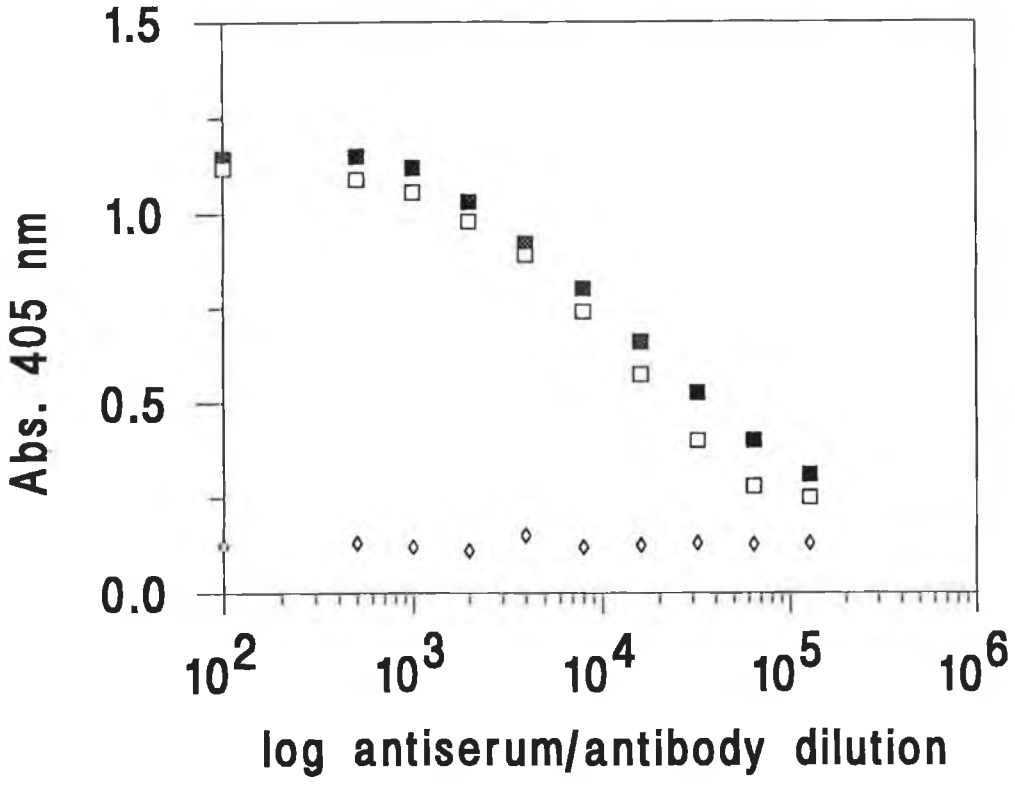
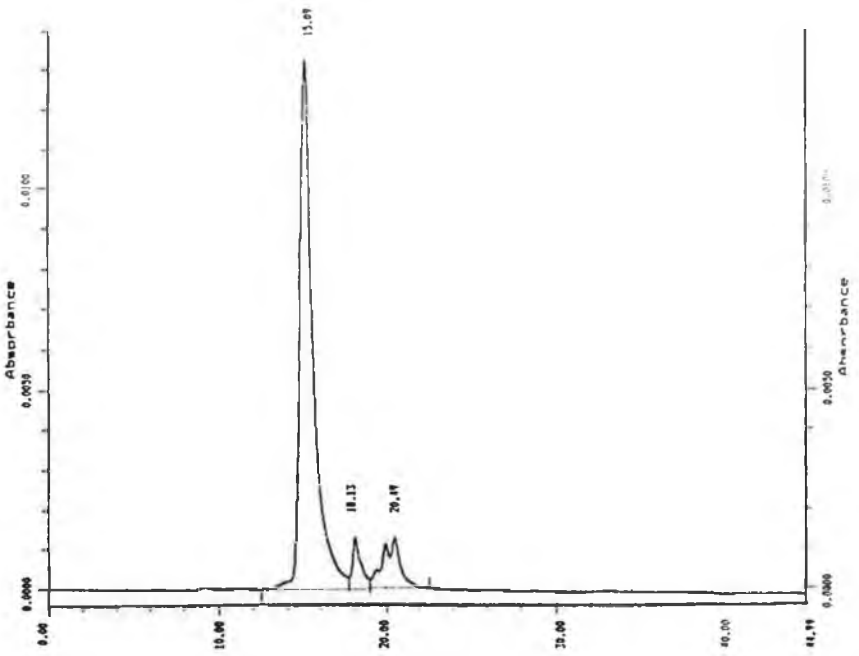


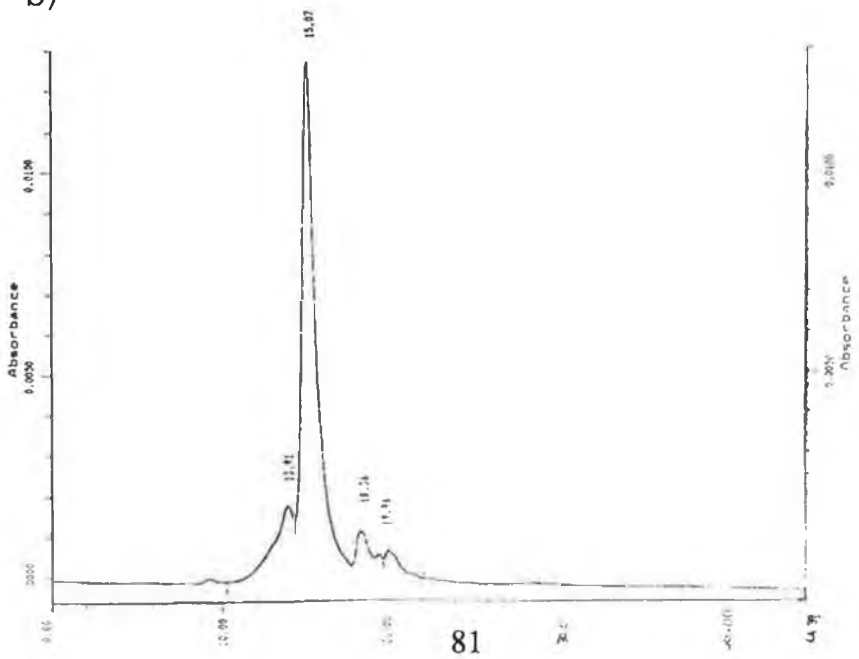
Figure 3.8.2

HPLC chromatograms of a) affinity-purified anti-CHLDH antibody and b) a commercially available affinity-purified antibody, anti-mouse IgG (Sigma), using a Phenomenex Biosep-Sec S4000 10 µm column (section 2.9.1). The mobile phase was 0.1M sodium phosphate buffer, pH 7.0, at a flow rate of 0.75 ml/min. Proteins were detected at 280 nm. The retention times of both antibodies was 15.08 min. The purity of the anti-CHLDH antibody compares favourably with the commercial antibody.

a)



b)



3.8.4 Characterisation of purified anti-CHLDH antibody by SDS-PAGE

The purified antibody was also characterised by SDS-PAGE electrophoresis, as outlined in section 2.8. The affinity-purified anti-CHLDH antibody and a commercially available affinity-purified goat anti-human IgG antibody, were electrophoresed under non-reducing conditions using a 5-20% gradient gel, and stained using Coomassie Brilliant Blue. The molecular weight markers are shown in lane 1 (Figure 3.8.3). Affinity-purified anti-CHLDH and affinity-purified anti-human IgG (M.W. 150000-180000 Da) are shown in lanes 3 and 4, respectively. BSA (M.W. ~66000 Da) was run in lane 2. The single band for the anti-CHLDH IgG indicates a highly purified preparation of IgG alone, which compares favourably with the commercial preparation. The higher molecular weight of the goat antibody over the rabbit antibody could be due to some species variability.

3.9 Conjugation of horse radish peroxidase (HRP) to anti-CHLDH antibody.

Affinity-purified anti-CHLDH antibody was conjugated to the enzyme, HRP, as described in section 2.5.2. The HRP-anti-CHLDH antibody conjugate and the unconjugated antibody were purified from the unconjugated enzyme by ammonium sulphate precipitation and diluted 1:1 with glycerol. The glycerol stabilises the conjugate during storage at -20°C, as it prevents ice formation.

3.9.1 Characterisation of HRP-anti-CHLDH antibody by HPLC

The HRP-labelled anti-CHLDH antibody was characterised by HPLC as described in section 2.9.1. A peak was obtained at a retention time of 9.49 min, indicating that a substance of molecular weight greater than IgG (which was shown to be represented by the peak at 15.10 min), was present (Figure 3.9.1). The molecular weight of the HRP-anti-CHLDH antibody conjugate will vary depending on the number of peroxidase molecules coupled to the antibody. Different levels of conjugation were observed between 9.49 and 15.10 min., indicated by the fact that the absorbance reading does not return to the baseline. The large peak seen at 19.63 min was shown to be due to the glycerol added, to stabilise the conjugate during storage. No free peroxidase was found in the sample. If any peroxidase was present, one would expect to find a peak between 15.10 (IgG, m.w. ~150,000 Da) and 19.63 (glycerol, m.w. ~80 Da) min, since the molecular weight of peroxidase is ~44,000 Da.

Figure 3.8.3

Analysis of the purity of the affinity-purified anti-CHLDH antibody by SDS-PAGE (5-20% gradient gel), under non-reducing conditions, and stained with Coomassie Brilliant Blue (section 2.8). Prestained molecular weight markers (Sigma SDS-7B), with molecular weights ranging from 32,000 - 195,000, were electrophoresed in lane 1. The molecular weight markers were triosephosphate isomerase (32,000), lactic dehydrogenase (35,000), fumarase (52,500), pyruvate kinase (63,000), fructose 6-phosphate kinase (84,000), β-galactosidase (112,000) and α_2-macroglobulin (195,000). Affinity-purified anti-CHLDH antibody and affinity-purified anti-human IgG antibody were electrophoresed in lanes 3 and 4, respectively. BSA was run in lane 2. The purity of the anti-CHLDH antibody (band found at molecular weight between 112 kDa and 195 kDa), compares favourably with the commercial preparation, indicated by the lack of contaminating protein bands.

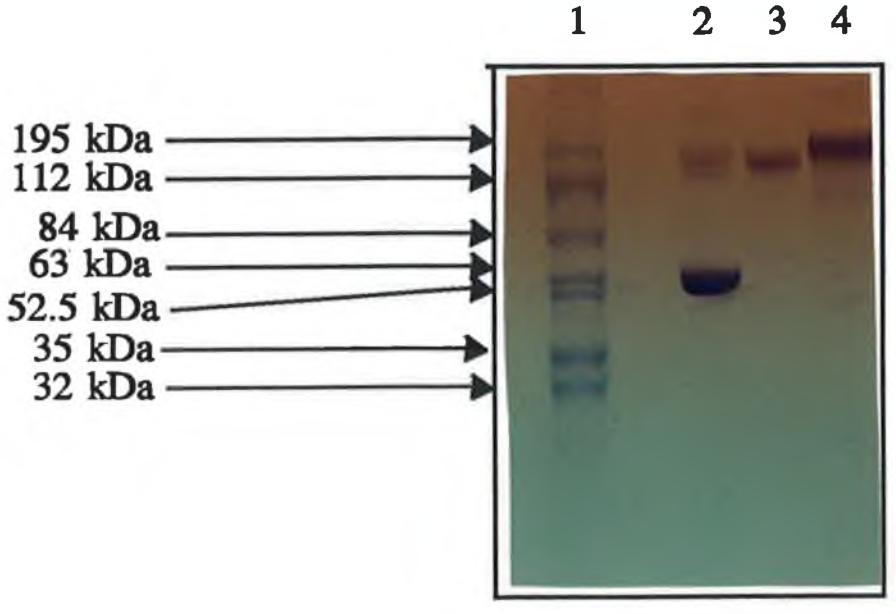
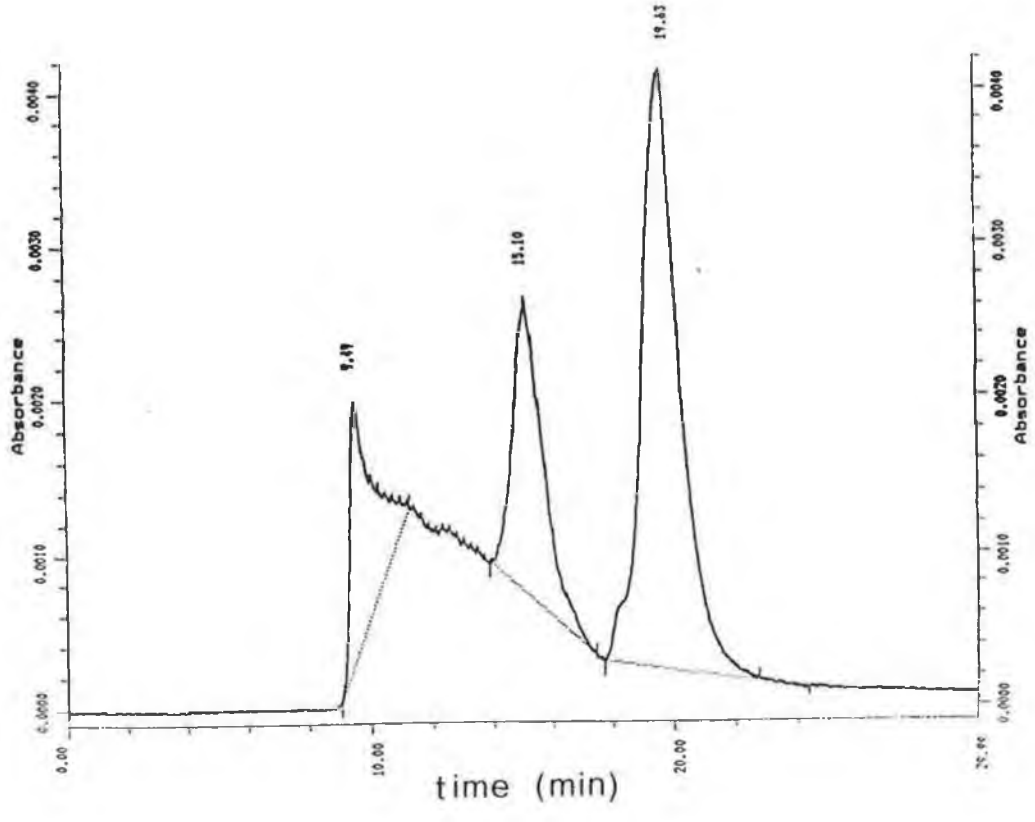


Figure 3.9.1

HPLC chromatogram of HRP-labelled anti-CHLDH antibody using a Phenomenex Biosep-Sec S4000 10 µm column. The mobile phase was 0.1M sodium phosphate buffer, pH 7.0, at a flowrate of 0.75 ml/min. Protein was detected at 280 nm. The presence of HRP-labelled anti-CHLDH antibody is indicated by the large protein peak at 9.49 min. Unconjugated anti-CHLDH antibody is also present in the sample, as indicated by the peak at 15.10 min. The large peak seen at 19.63 min was shown to be due to the glycerol added to stabilise the conjugate during storage.



3.9.2 Determination of the working dilution of HRP-labelled anti-CHLDH antibody

The working dilution of the HRP-anti-CHLDH antibody was determined, for use in an ELISA, as described in section 2.5.3. Serial dilutions (1:50 - 1:25600) were prepared in 0.15M PBS, pH 7.4. The working dilution of the conjugate was determined to be 1:4000 (Figure 3.9.2).

3.10 Determination of CHLDH by two-site sandwich ELISA

Samples of CHLDH were prepared at concentrations ranging from 0.005-10 µg/ml, in 0.15M PBS, pH 7.4 and used in the ELISA. The optimal coating antibody concentration was determined to be 5 µg/ml, as higher antibody concentrations did not give increased binding to the plate. The HRP-anti-CHLDH second antibody was used at its working dilution of 1:4000. Negative controls (heart LDH from rabbit, porcine and bovine sources) were included. PBS alone was added to some control wells.

The ELISA was carried out as described in section 2.5.4 and the results plotted on a linear/log scale. The standard curve shown in Figure 3.10.1. The curve is linear between 0.020-0.625 µg/ml (Figure 3.10.2).

3.11 Labelling of anti-CHLDH antibody with fluorescein

Affinity-purified anti-CHLDH antibodies were conjugated with fluorescein isothiocyanate, a functionalised derivative of fluorescein according to the method of Goding (1984), as outlined in section 2.6.1. The conjugate was purified by gel filtration on a Sephadex-G25 column. The fluorophore to protein ratio, F/P, (i.e. the number of FITC molecules per antibody molecule), was estimated by the absorbance method using the formula given in section 2.6.1. (The and Feltkamp, 1970). The F/P was determined to be between 3-5, for all the conjugate preparations undertaken.

Figure 3.9.2

Determination of the working dilution of HRP-anti-CHLDH antibodies. Serial dilutions (1:50 - 1:25600) of the conjugate were prepared in 0.15M PBS, pH 7.4 and titred as outlined in section 2.5.3. The working dilution was determined to be 1:4000, as this was the dilution which gave an absorbance value of half of the lowest dilution (1:50) absorbance value.

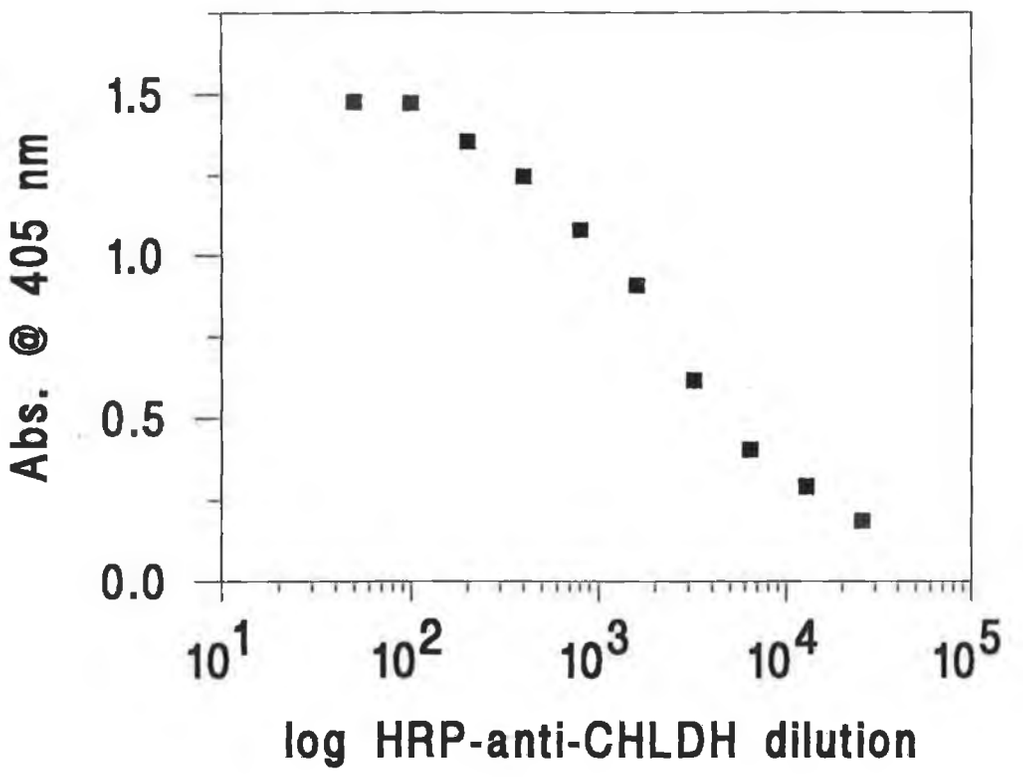


Figure 3.10.1

Determination of the linear range of the two-site sandwich ELISA for CHLDH. Samples (0.005 - 10 µg/ml CHLDH) were prepared in 0.15M PBS, pH 7.4, and the ELISA was performed as outlined in section 2.5.4. The second antibody, HRP-labelled anti-CHLDH antibody, was used at its working dilution of 1:4000. Absorbance values were plotted against the log CHLDH concentration. The linear range was determined to be between 0.0200 - 0.625 µg/ml (see figure 3.10.2).

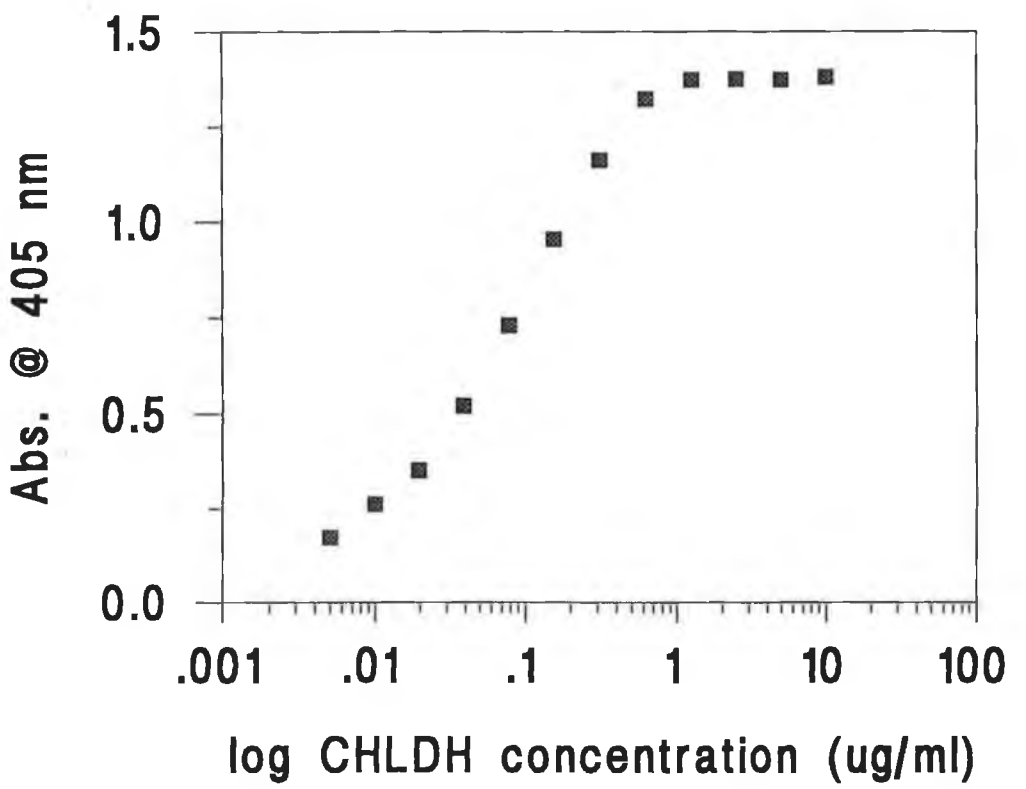
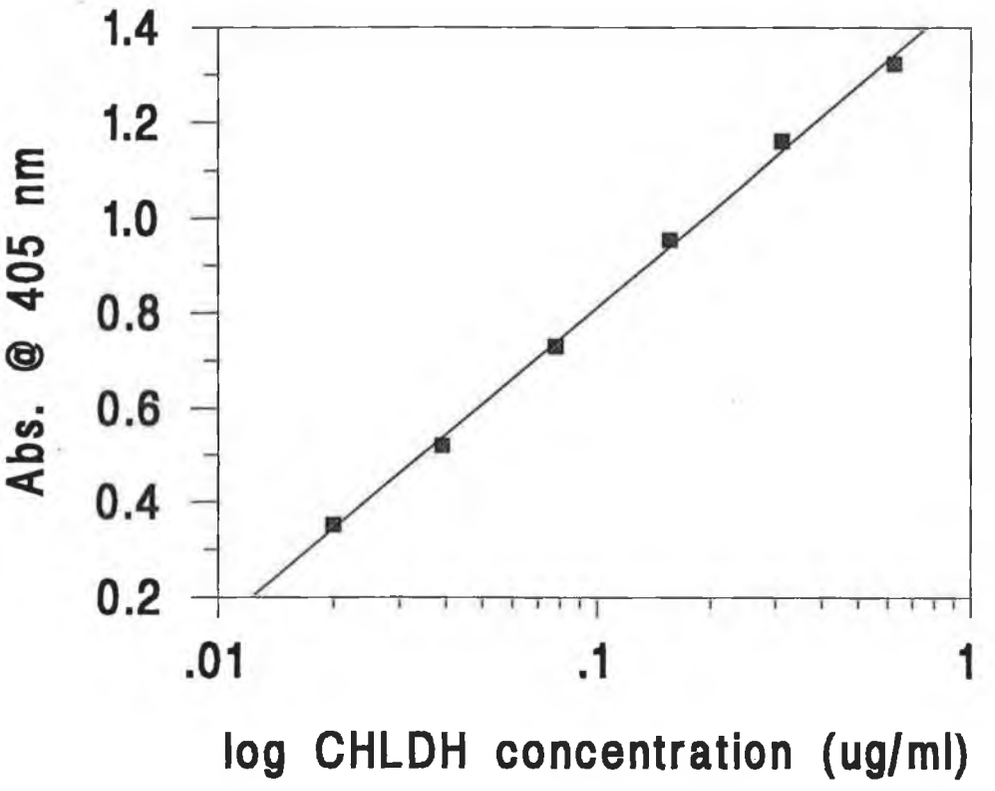


Figure 3.10.2

Linear range of two-site sandwich ELISA for CHLDH. Absorbance values were plotted against the log CHLDH concentration. The linear range is between 0.020 and 0.625 µg/ml (r=0.999).



3.11.1 Characterisation of FITC-anti-CHLDH antibody by spectral analysis

Unconjugated IgG (anti-CHLDH antibody), i.e. IgG sample which was subjected to the conjugation procedure in the absence of FITC, and FITC-anti-CHLDH antibody were diluted in 0.15M PBS, pH 7.4, and scanned from 250-550 nm using the Shimadzu UV/vis spectrophotometer. The resulting scans are shown in Figure 3.11.1. The IgG spectra has an absorbance peak with a maximum at 280 nm, whereas the FITC-IgG conjugate has the same absorbance peak at 280 nm but also an absorbance peak at 495 nm, the absorbance maximum of fluorescein.

3.11.2 Characterisation of FITC-anti-CHLDH antibody by HPLC with PDA detection

The FITC-IgG conjugate and unconjugated IgG were characterised by gel permeation HPLC with photodiode array (PDA) detection, as outlined in section 2.9.2. The peak retention time of IgG using the protein Pak SW 300, 10 µm column, was found to be approximately 15.5 min. All the peaks which were initially detected at 280 nm, were scanned from 220-600 nm, and the resulting contour pattern recorded (Figure 3.11.2). The IgG sample produced a single absorption peak centred at 280 nm. Absorbances below 250 nm are due to buffer solutions. The peak found at 22.5 min is due to the added preservative, thiomerosal. The contour plot for the conjugate sample shows an extra absorbance peak centred at 495 nm, at the same retention time as IgG. This proves that the FITC is conjugated to the protein. FITC has a very low molecular weight, 389.4 Da, so if it was not conjugated to the protein one would expect it to have a retention time >22 min.

Figure 3.11.1

UV/visible scans of a) unconjugated anti-CHLDH antibody b) unconjugated FITC and c) FITC-anti-CHLDH antibody. The samples were prepared in 0.15M PBS, pH 7.4 and scanned from 250-550 nm, using the Shimadzu UV/vis spectrophotometer. The scan of unconjugated anti-CHLDH antibody shows a protein absorbance peak at 280 nm, while the FITC-labelled anti-CHLDH antibody shows a protein peak at 280 nm and the appearance of a new peak at 495 nm. This peak at 495 nm was shown to be due to FITC.

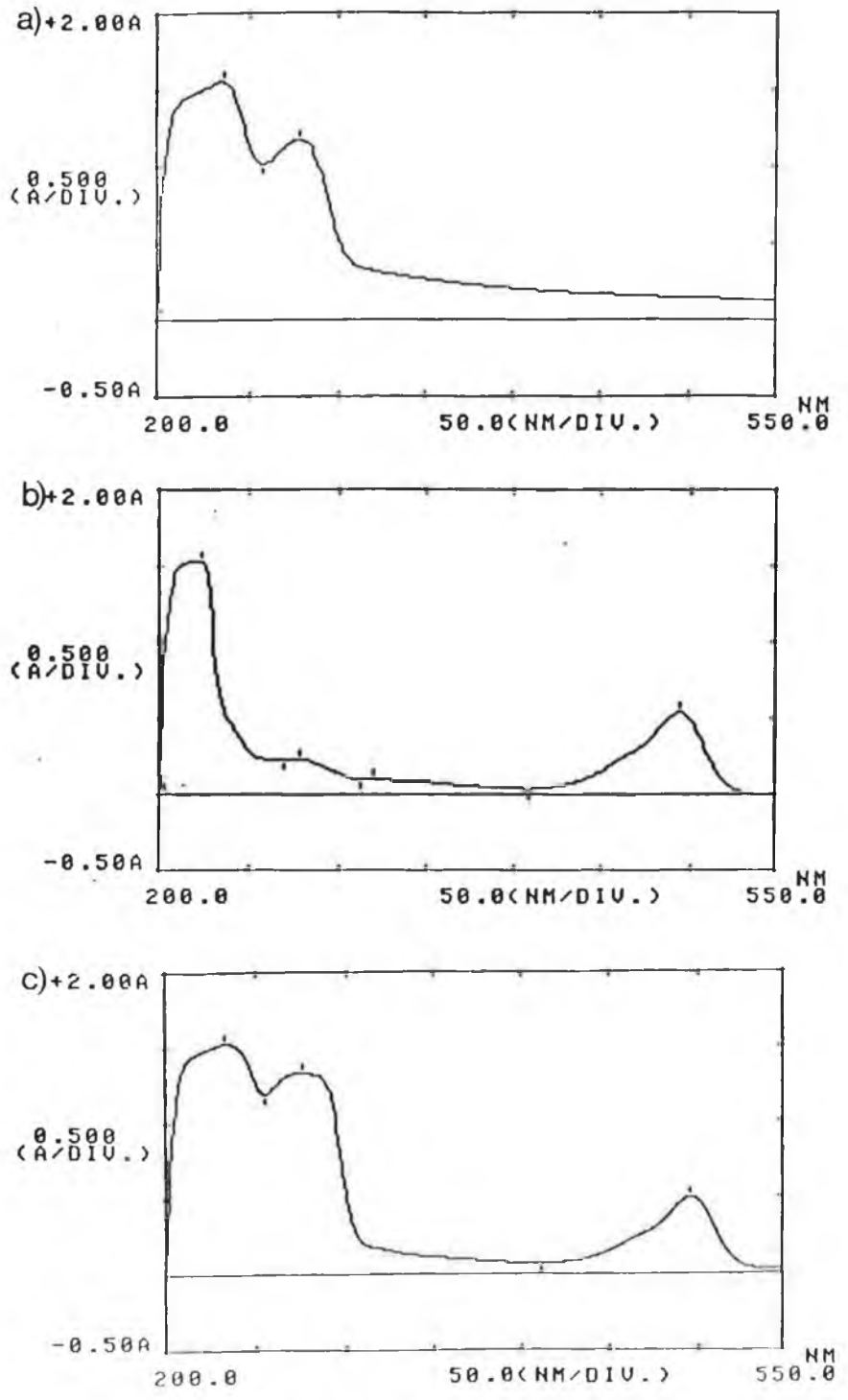
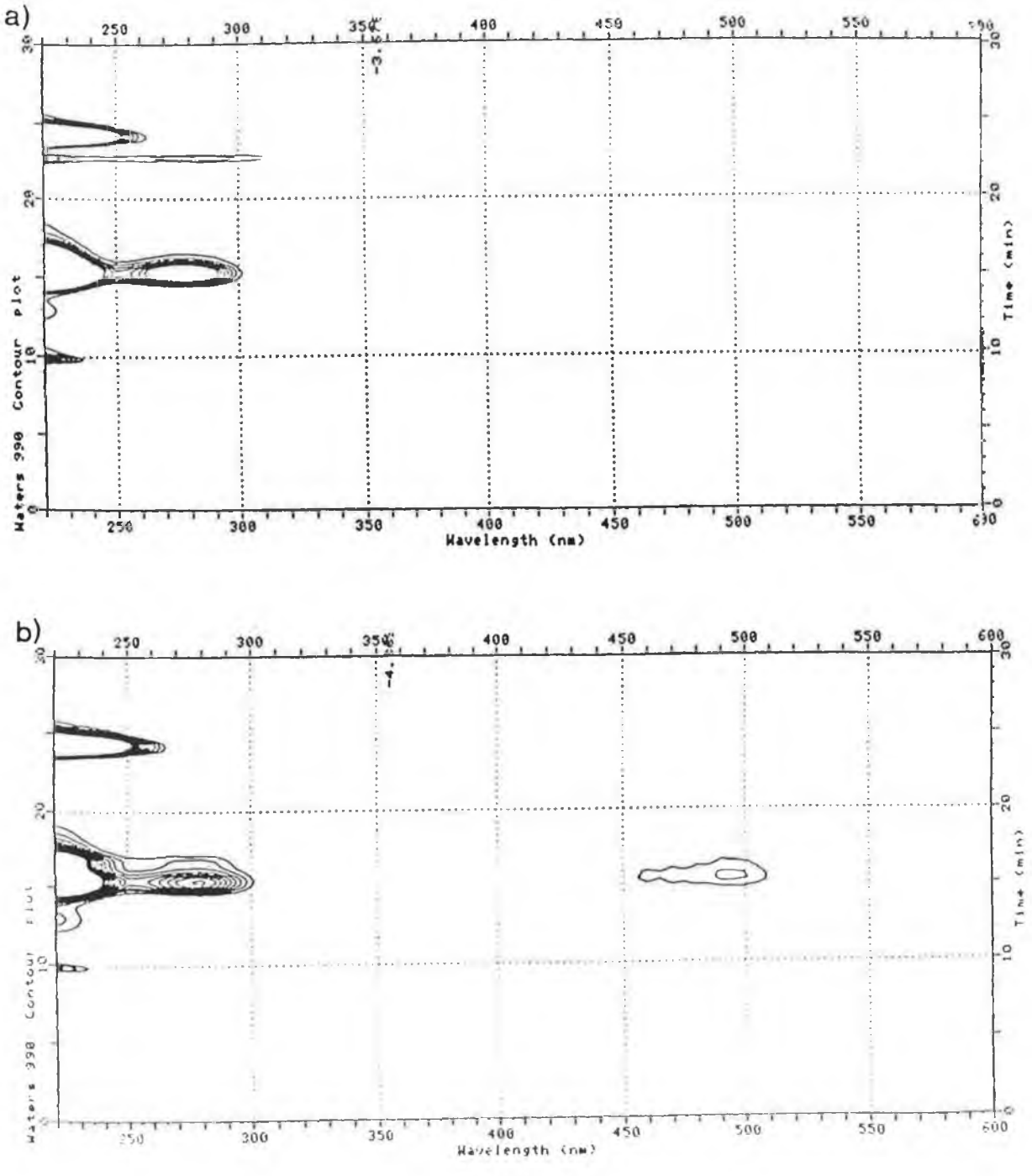


Figure 3.11.2

HPLC chromatogram contour pattern of a) unconjugated anti-CHLDH antibody and b) FITC-anti-CHLDH antibody, with PDA detection (section 2.9.2). All the peaks detected initially at 280 nm were scanned from 220-600 nm. The unconjugated antibody sample produced a single absorption peak at 15.5 min, while the FITC-labelled anti-CHLDH antibody produced two peaks at the same retention time, one at 280 nm and the other centred at 495 nm. This proves that the FITC is conjugated to the antibody.



3.11.3 Determination of the working dilution of FITC-anti-CHLDH antibody

The working dilution of the conjugate for use in immunoassays, was determined as described in sections, 2.6.3 and 2.5.3. Serial dilutions 1:25-1:6400 were prepared in 0.15M PBS, pH 7.4, and added to CHLDH-coated fluorimeter microtitre plates (Dynatech). The fluorescence intensity of the contents of each well was read using the microtitre plate reader facility of the Perkin-Elmer fluorimeter, with the excitation and emission wavelengths set at 488 nm and 520 nm, respectively. The excitation and emission slit widths were set at 10 nm. Unconjugated antibody (anti-CHLDH antibody) and FITC-labelled non-specific antibody (FITC-anti-mouse IgG) were included as controls. The working dilution of the FITC-anti-CHLDH antibody was determined to be between 1:100-1:150, as this was the dilution which gave a fluorescence intensity of half the lowest dilution (1:25), fluorescence intensity value (Figure 3.11.3).

3.11.4 Fluorescence-linked immunosorbent assays

One-step (antigen and labelled-antibody premixed before adding to the immobilised antibody) and two-step (antigen and labelled-antibody added at separate times to the immobilised antibody) fluorescence-linked immunosorbent assays were performed in Dynatech fluorimeter microtitre plates. These assays were performed to assess the labelled antibodies in the immunoassays, and to compare the one-step and two-step assays in terms of sensitivity and range.

3.11.4.1 One-step fluoroimmunoassay

Antigen (CHLDH) concentrations in the range 0.015 - 10.0 µg/ml, were premixed with a 1:100 dilution of FITC-anti-CHLDH antibody (working dilution as determined in section 3.11.3), and added to the anti-CHLDH antibody coated plate (section 2.6.3.1), in triplicate. Following a washing step, the fluorescence was determined, as outlined in section 3.11.3. The standard curve is shown in Figure 3.11.4. The curve is linear between 0.5 - 2.0 µg/ml (r=0.970). Above 2.0 µg/ml, the second labelled antibody is no longer in excess, and so a decrease in fluorescence is observed.

3.11.4.2 Two-step fluoroimmunoassay

The two step fluoroimmunoassay was performed under the same conditions as the one step assay, except that the CHLDH and the anti-CHLDH antibody were incubated with

the plate separately, with a washing step between each incubation (section 2.6.3.2). The standard curve is shown in Figure 3.11.4. The curve is linear between 0.125 - 4.0 µg/ml (r=0.993). Above 4.0 µg/ml, the curve levels off, indicating that the capture antibody is saturated with antigen.

Figure 3.11.3

Determination of the working dilution of FITC-labelled anti-CHLDH antibody. Serial dilutions of the labelled antibody were prepared in 0.15M PBS, pH 7.4, and titred as outlined in sections 2.6.3 and 2.5.3. The fluorescence intensity was read at excitation and emission wavelengths of 488 nm and 520 nm, respectively, with the excitation and emission slit widths set at 10 nm and 10 nm, respectively. The fluorescence intensity was plotted against the log FITC-anti-CHLDH antibody dilution. The working range was determined to be 1:100 - 1:150, as this was the dilution range which gave a fluorescence intensity value of half the lowest dilution (1:25) fluorescence intensity value.

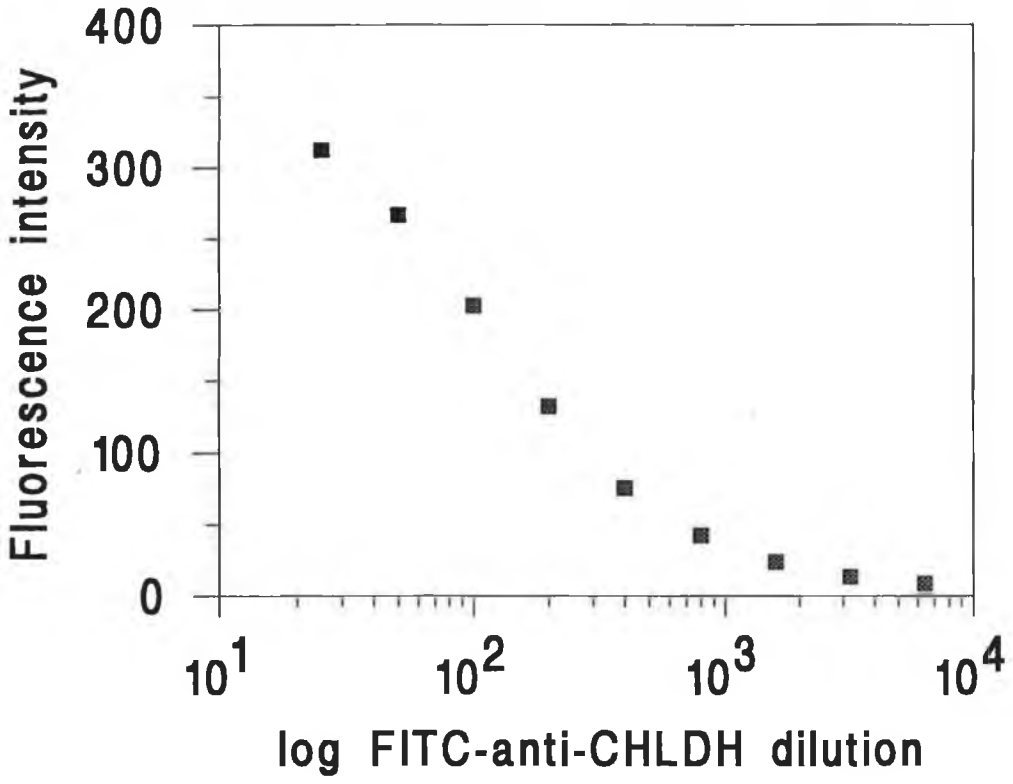
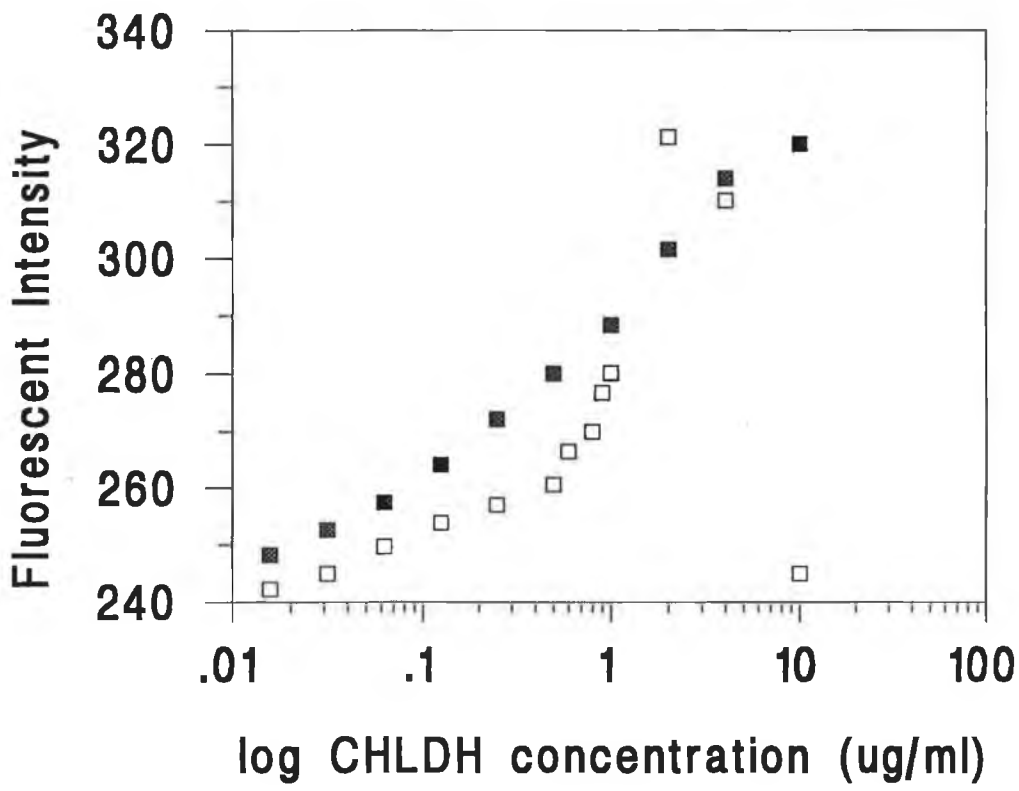


Figure 3.11.4

One-step (■) and two-step (□) fluorescence-linked immunosorbent assays (section 3.11.4), performed on fluorimeter microtitre plates. Antigen (CHLDH) samples, in the range 0.015 - 10.000 µg/ml, were prepared in 0.15M PBS, pH 7.4, and the assays performed as outlined in section 2.6.3.1 and 2.6.3.2., using a 1:100 dilution of FITC-anti-CHLDH antibody. Fluorescence intensity values were read at excitation and emission wavelengths of 488 nm and 520 nm, respectively, with the slit widths set at 10 nm. The intensity values were plotted against log CHLDH concentration. The linear range of the one-step assay was 0.5 - 2.0 µg/ml (r=0.970) and 0.125 - 4.000 µg/ml (r=0.993) for the two-step assay.



3.12 Discussion

This chapter outlines the production, purification and characterisation of polyclonal antibodies against LDH, isolated from chicken heart muscle. The purified antibodies were subsequently labelled with HRP, for enzyme-linked immunosorbent assay development, and labelled with FITC, for use in the development of a model evanescent wave fibre optical immunosensor (Chapter 4).

The antigen, CHLDH, is a large molecular weight protein (Cahn *et al.*, 1962), and so does not require crosslinking to a carrier molecule for production of an immune response. A high titre polyclonal sera was obtained, 1:32000, after repeated immunisations. The antiserum contains antibodies of different affinities against different epitopes on the CHLDH surface. The antibodies were purified by immunospecific affinity chromatography, resulting in specific antibodies only, against the CHLDH. This method of purification was feasible as the CHLDH is inexpensive, and commercially available in a purified form. Immunospecific affinity chromatography (Eveleigh and Levy, 1977) requires large quantities of purified antigen and so preparation of an affinity column is not feasible for all antigens. Other methods of purification such as affinity chromatography using protein A, which binds to the Fc portion of class G immunoglobulin (Underwood *et al.*, 1983), or Affi-T columns, a matrix with an immobilised thiophilic ligand which binds to the disulphide linkages in the IgG molecule (Yurov *et al.*, 1994), isolate the immunoglobulin fraction of an antiserum, which contains both specific and non-specific (host) antibody.

A two-site sandwich enzyme-linked immunosorbent assay (ELISA), was developed using HRP-labelled anti-CHLDH antibody as the second antibody. The sandwich assay system is simple, specific and sensitive (Catty and Raykundalia, 1988). The linear range scans two orders of magnitude (0.020 - 0.625 µg/ml), but the absorbance change was large for small changes in concentration. The sensitivity of a two-site ELISA depends on the surface concentration of the immobilised antibody, the purity of the immunoreactants and the concentration of the labelled species. The fact that the antibodies are immunospecifically affinity-purified ensures that the surface density of specific antibody is high.

Often two-site sandwich ELISAs are performed employing high affinity monoclonal antibodies as the solid phase immobilised antibody. After exposing the immobilised antibody to the test sample, and following washing, the immunocomplex is exposed to

another monoclonal antibody which binds a different (Corti *et al.*, 1994) or repeated but spatially distant epitope on the same antigen (Wang *et al.*, 1994). Monoclonal antibodies are expensive and time-consuming to produce, however the sensitivity and specificity of immunoassays using monoclonal antibodies is very high. The disadvantage of the double monoclonal system is that there must be two target epitopes on opposite sites of the antigen, to facilitate detection. Alternatively, the second antibody can be polyclonal and so bind to several epitopes on the antigen surface (Akhoundi *et al.*, 1994), thereby increasing sensitivity. The ELISA developed here however, employed the same polyclonal preparation as the capture and labelled antibody. The antigen is large and so has several epitopes spatially separated over its surface. The fact that the antibody was affinity purified increases the sensitivity of the assay, as all of the capture antibody is specific for the CHLDH. It is difficult however to purify the high avidity fraction of the antibody, without denaturing the binding sites.

The purified antibodies were assessed for purity by HPLC using a gel permeation column and SDS-PAGE. Both methods separate the molecules in a sample on the basis of size, and the number of peaks (HPLC) or bands (SDS-PAGE) detected gives information on the relative purity of the sample. The largest molecular weight molecule elutes first from the HPLC gel permeation column, and so high molecular weight molecules have shorter retention times than low molecular weight molecules (Carty and O'Kennedy, 1988). The affinity-purified anti-CHLDH antibody was shown to be relatively pure since only one strong peak was visible at a retention time of 15.08 min. The smaller peaks were shown to be due to the added preservative, thiomerosal and glycine salts. Glycine buffer was used to elute the specific antibody from the affinity column. The preparation was not dialysed sufficiently to remove all the glycine salts.

SDS-PAGE allows visualisation of the purity of the antibody preparation, by staining the separated proteins, with a protein specific dye, Coomassie Blue. The proteins are separated in the gel on the basis of size, by molecular sieving through the pores in the gel (Laemmli, 1970). The smaller molecules move further through the gel. The separation can be improved by varying the % acrylamide in the gel, which varies the pore size of the gel matrix. Only one blue band was visualised on the gel indicating that the purification yielded only immunoglobulin. There is a slight difference in molecular weight of the immunoglobulin from the rabbit and goat (commercial) sources. This was not unexpected, since the molecular weight of IgG from different animal species is

slightly different. This is probably due to the different ratios of the κ and λ light chain types in different animal species (chapter 6, Tijssen, 1985).

The anti-CHLDH antibody was labelled with a derivative of fluorescein (FITC) for use in the development of the evanescent wave fibre-optic immunosensor. The conjugate was characterised by gel permeation HPLC with photodiode array detection (Escribano *et al.*, 1988). The photodiode array generates an absorption spectrum over a range of wavelengths at specified time intervals (typically every 0.5 second). Unlabelled anti-CHLDH antibody and FITC-labelled antibody was passed through the column and the contour plots reveal an extra absorption peak between 460 - 510 nm, for the FITC-labelled antibody.

One-step and two-step two-site sandwich fluoroimmunoassays were performed using the FITC-labelled anti-CHLDH antibody at its working dilution of 1:100. In the one-step assay, the CHLDH and FITC-anti-CHLDH antibody were premixed, allowing the antigen and labelled antibody to bind in solution, before incubating with the capture antibody. The one-step assay is simpler and quicker to perform as the analyte and labelled antibody are added in one step. Corti *et al.*, (1994), performed a one-step immunoassay for the quantitation of tumour necrosis factor (TNF) α. The immunoassay, however, employed monoclonal antibodies against different epitopes. The anti-CHLDH antibody is polyclonal in nature and therefore binds to several epitopes on the antigen surface. The antigen is a large molecule and so for a limited concentration range there are enough epitopes left free to bind to the capture antibody. The rate at which the immunocomplex binds to the immobilised antibody would be reduced, however, due to steric hindrance and diffusional effects.

The linear range of the two-step assay is wider than that of the one-step assay. It is clear from Figure 3.11.4, that the one-step assay is sensitive within a very limited linear range. At high concentrations of antigen, the labelled antibody is no longer in excess and so a decrease in the fluorescence is observed. This 'high-dose hook effect' can be shifted to higher antigen concentrations by using a lower dilution of labelled antibody. This may result in higher background fluorescence and reduce the sensitivity at the lower end of the antigen concentration scale, as the number of free epitopes for low concentrations would be reduced.

In conclusion, antibodies against the CHLDH were produced, immunospecifically purified and labelled with FITC, for the development of the evanescent wave fibre optic

immunosensor described in chapter 4. An ELISA system was developed and was used for model fibre ELISAs (chapter 4), to assess the optimal coating concentration etc. of the fibre probes. The feasibility of performing one-step and two-step fluoroimmunoassays with the same batch of polyclonal antibodies was also investigated.

CHAPTER 4

DEVELOPMENT OF A FIBRE OPTICAL FLUOROIMMUNOSENSOR BASED ON THE EVANESCENT WAVE TECHNIQUE

4.1 Introduction

This chapter decribes the development of an antibody-based optical biosensor. Chicken heart LDH was used as the analyte in this model system. The fluorescently-labelled immunoassay system was performed on the exposed core of optical fibres, and the evanescent wave was used to probe the reaction. The binding of antibody and antigen was monitored in 'real-time'. The fibre-based immunoassay was optimised using ELISA techniques. The evanescent wave optical biosensor was compared to a commercially available biosensor based on surface plasmon resonance, the BIAcore™.

4.2 The evanescent wave

When an electromagnetic wave (light ray), is incident on the interface between two dielectric media, having refractive indices, n_1 and n_2, such that $n_1 > n_2$, and when the light ray angle, α, is greater than the critical angle, α_c, total internal reflection results, with no refracted light entering the lower refractive index medium (Figure 4.1(a)). The critical angle of the waveguide is determined by the refractive indices of the two media, according to the following formula (Love *et al.*, 1991):

$$\alpha_c = \sin^{-1}\left(\frac{n_2}{n_1}\right) \qquad (4.1)$$

An electromagnetic wave analysis of this situation yields a more complete picture. In the denser medium, the superposition of the incident and the reflected light wave, results in the generation of an electromagnetic standing wave (Figure 4.1(b)). In addition, an electromagnetic wave extends a short distance, typically 0.1 - 1.0 μm ($<\lambda_o$, the wavelength of the incident light in a vacuum), into the lower refractive index medium (n_2). This non-propagating electromagnetic radiation is called the 'evanescent wave'. The amplitude of the evanescent wave is non-zero for distances close to the interface surface, but decays exponentially to zero with distance, δ, from the waveguide surface, although there is no net flow of energy across the interface.

The exponential decay is depicted in Figure 4.1(b). The distance, d_p, the penetration depth, is the distance for the electric field amplitude of the evanescent wave to fall to 1/e or 37% of its value at the waveguide surface. It is defined by the following formula:

$$d_p = \frac{\lambda_0}{2\pi(n_1^2 \sin^2 \alpha_1 - n_2^2)^{\frac{1}{2}}} \qquad (4.2)$$

The penetration depth is dependent on the vacuum wavelength (λ_0), of the totally internally reflected light, the refractive indices of the two media, n_1 and n_2, and the incident light angle, α_1 (Love *et al.*, 1991). From the formulas, 4.1 and 4.2, it is clear that the penetration depth rises sharply as α_1 approaches the critical angle, α_c.

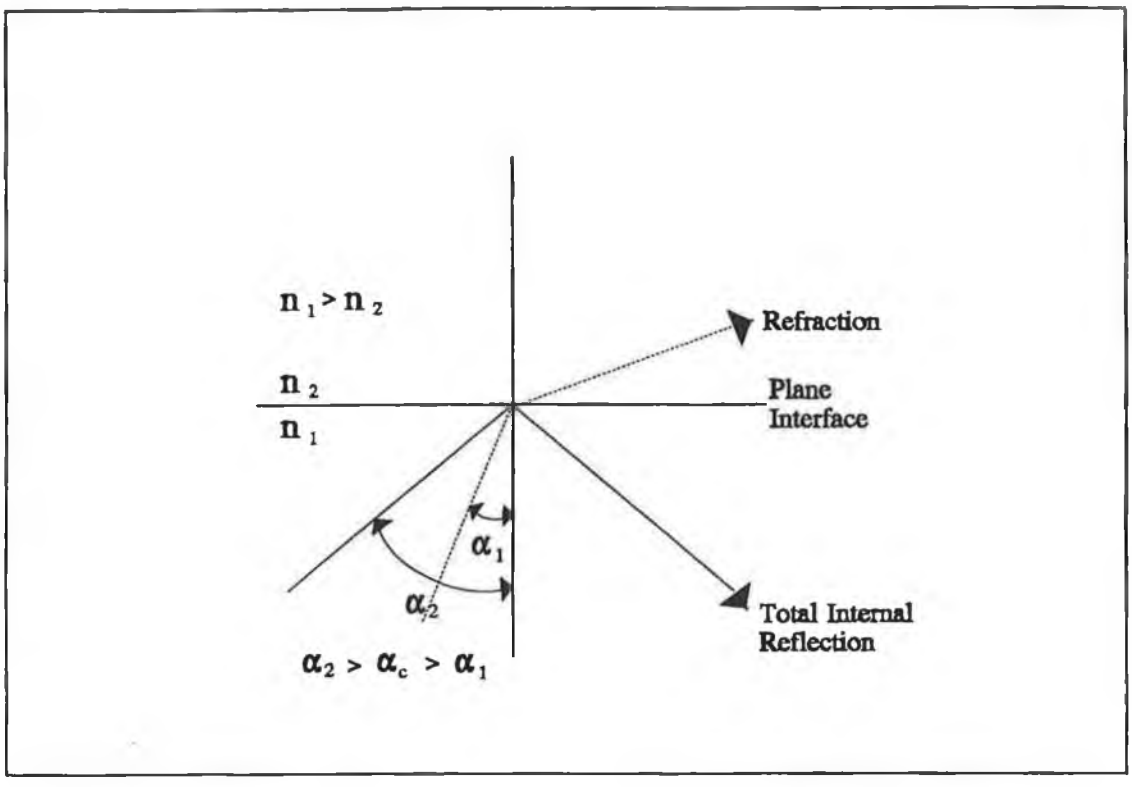


Figure 4.1(a): Plane wave total internal reflection and refraction at a dielectric interface

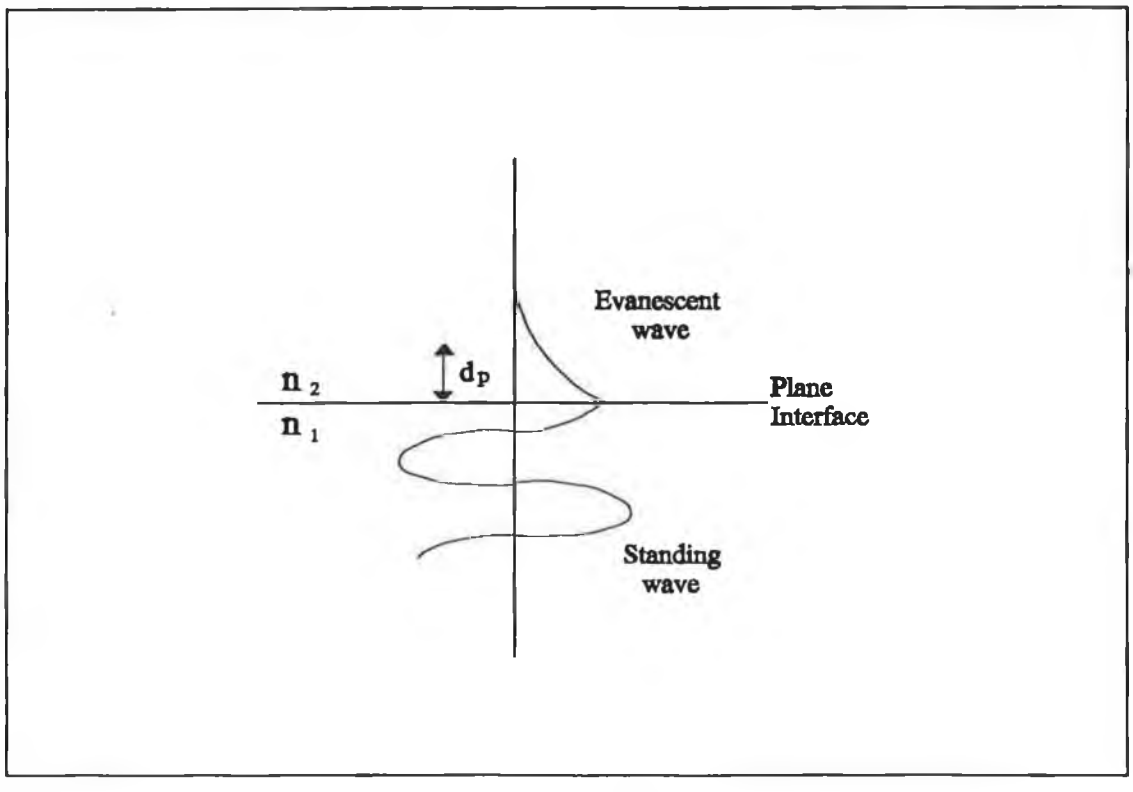


Figure 4.1(b): Diagrammatic representation of the standing wave generated as a result of incident and reflected light interference and its associated evanescent wave

4.3 Fluorescence

A molecule in its ground state may be raised to a higher energy level or excited state by absorption of photons at particular wavelengths. When a molecule is in this excited state, it is unstable and returns to the ground state by spontaneous emission of a photon. The emitted photon, called fluorescence, is generally of a lower energy than that of the absorbed photon, due to the energy loss (e.g. release of heat), encountered during internal conversion. The nature of fluorescence, which is a property of relatively few molecules, termed fluorophores, is discussed in Ward and Fothergill (1976). The excitation and emission scans for fluorescein isothiocyanate (FITC), are shown in Figure 4.2.

Fluorescence intensity can be related to fluorophore concentration as follows;

$$I_f = \phi I_0 (1 - 10^{-\varepsilon L c}) \qquad (4.3)$$

where

ϕ = fluorescence quantum efficiency

I_o = incident radiation intensity

ε = molar extinction coefficient

L = optical path length

c = fluorophore concentration

The *fluorescence quantum efficiency,* ϕ, is a property of the fluorophore and is a measure of the ratio of photons emitted to those absorbed, and is always <1.0, but more usually <0.1. Fluorescein has a high quantum efficiency, ~0.9, in its natural state, but this is somewhat reduced on conjugation to proteins, ~0.4. The *molar extinction coefficient,* ε, is a measure of the strength of absorption at the maximum absorbance wavelength of the fluorophore, e.g. fluorescein has a high molar extinction coefficient of 80,000 $cm^{-1}M^{-1}$. Exciting a fluorophore at a wavelength away from the maximum absorbance wavelength, has the same effect as using a more weakly absorbing fluorophore. The maximum fluorescence peak wavelength is a characteristic of the fluorophore and remains the same regardless of the excitation wavelength.

The environment which surrounds the fluorophore can effect its fluorescence intensity, by changing the stability of the excited state. Environmental factors include the nature of the solvent i.e. ionic composition, pH and temperature. The loss of energy in the

excited state through vibrational energy transfer to the environment before emission results in the *Stoke's shift*, which is an intrinsic property of the fluorophore. It is defined as the difference in wavelength between the excitation and emission maxima, and for fluorescein is approximately 30nm. The longer the Stoke's shift of a fluorophore, the less overlap there is between the excitation and emission spectra. This is important for evanescent wave sensing where the excitation radiation must be separated from the fluorescence (Haugland, 1991).

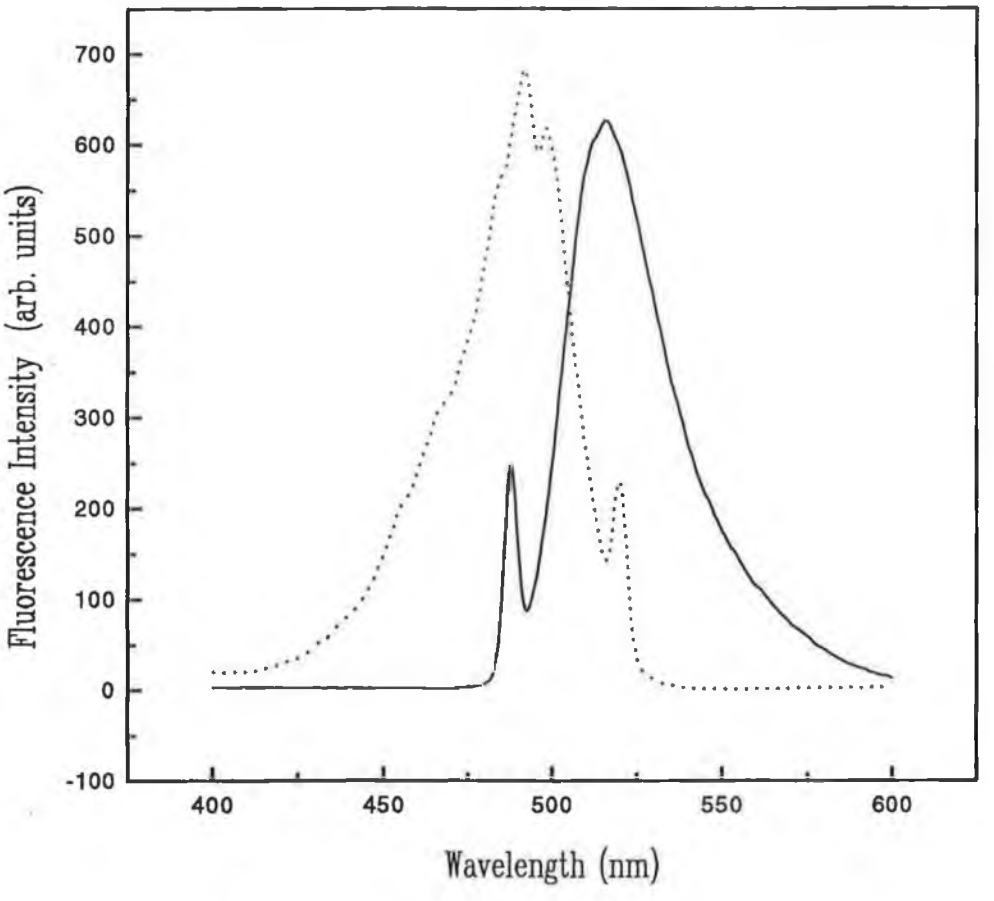
Reduction of fluorescence emission results from different physical and chemical phenomena such as the inner filter effect, photobleaching and quenching. The inner filter effect occurs at high concentrations of fluorophore, where part of the emission intensity is reabsorbed by other fluorophore molecules, resulting in a plateau effect. Photobleaching is not a completely understood phenomenon. It is believed that the absorption of high intensity light results in the irreversible destruction of the chemical structure of the fluorophore. Schauenstein *et al.* (1982), studied the effect of high intensity radiation on FITC-antibody conjugates, and showed increased photobleaching of fluorescein on conjugation to proteins.

Quenching is a phenomena resulting from an extrinsic process, which depopulates the excited state without the emission of a photon. The reduction of fluorescence results from the interaction of the fluorophore with other factors present in the system, such as temperature, solvent, concentration of fluorophore, impurities and oxygen. This property of fluorescent molecules has been exploited for the development of sensors, e.g. O_2 quenches the fluorescence of some ruthenium derivatives (O'Keeffe *et al.*, 1995)

4.4 Evanescent wave interactions

Normally there is no net flow of evanescent wave energy across the interface between two dielectric media such as the core/cladding interface of an optical fibre. However, if the optically less dense medium contains an absorbing species, within the penetration depth of the evanescent wave, a net energy loss from the reflecting wave, through interaction with the evanescent wave results. This is referred to as attenuated total reflection or ATR (Place *et al.*, 1985). The light beam does not pass directly into the absorbing species matrix, as the interaction takes place at the point of total internal reflection in the denser medium.

Figure 4.2: Excitation and emission profile for FITC. FITC has a maximum absorbance peak at 488 nm (....) and a maximum fluorescence peak at 520 nm (___). The Stoke's shift, the difference between the excitation and emission maxima is ~30 nm, and the quantum yield, the ratio of the emission to absorbance peak intensity, is ~0.9.



As the magnitude of the penetration depth of the evanescent wave is less than 1 μm, and the electrical intensity decreases exponentially with depth, the evanescent wave only interacts with molecules close to the interface surface, and those in the bulk solution remain relatively unaffected. If the absorbing species dissipates the absorbed energy as fluorescence, then the evanescent wave may be employed as an excitation source. A fraction of the fluorescent radiation emitted in the less dense medium, is coupled back into the optically denser medium, according to the principle of optical reciprocity. This principle states that the fluorescent radiation is coupled back into the waveguide in the same way as the inverse process, and has the same angular distribution and dependence on index, wavelength and distance, as the plane wave which generates an evanescent wave. The coupled fluorescence will propagate as a plane wave above the critical angle, within the higher refractive index medium. Love and Button (1988), showed experimentally and theoretically, that the total fluorescent signal obtained from one end of a short length of fibre surrounded with fluorescent material can be described by;

$$I_f \propto \varepsilon I_0 L a \left(\frac{r_{max}}{a}\right)^2 \sin^8\theta_{max} (NA)^{-4} \qquad (4.4)$$

ε = extinction coefficient of the fluorophore

I_o = excitation intensity of launched light

L = fibre sensing length

r_{max} = radius of the launch spot

θ_{max} = maximum external angle of the launch and acceptance cone

a = fibre core radius

NA = numerical aperture of the fibre

This expression emphasis the critical effect of high angle light in a fibre or waveguide ($\sin^8\theta$).

4.5 Optical fibres and evanescent wave sensing

A plastic clad optical fibre is a thin rod which typically consists of a transparent silica-based fibre core, of refractive index, n_1, surrounded by a plastic cladding of lower refractive index, n_2. Light launched down the optical fibre at angles greater than the

critical angle, α_c, of the waveguide, is propagated through the core by total internal reflection, as described in section 4.2. An evanescent wave is generated at the dielectric interface which extends into the plastic cladding. Optical fibres are used widely in the communications industry, as they allow transmittance of light over long distances, with very high efficiency.

If the cladding of the optical fibre is removed, we have a situation where a molecular species in solution can interact with the evanescent wave, as the sample solution now acts as the 'cladding'. Exposed core optical fibres can therefore be used for sensing. Most evanescent wave sensors cited in the literature are based on fluorescence detection. The evanescent wave radiation at the appropriate wavelength, excites fluorescent molecules either immobilised on or very close to the fibre core surface, thus optically separating the fluorescent molecules within the penetration depth from those in the bulk solution. The resulting fluorescence is coupled back into and propagated by the same optical fibre in higher order modes, and can be detected at the distal (Sutherland *et al.*, 1984), or proximal end (Hirschfeld and Block, 1984), of the fibre using appropriate filters and detectors. This effectively means that the fluorescence can be monitored at the output of the waveguide in the same optical plane as the excitation light. The separation of the fluorescence in the 'sensing volume' from that in the bulk solution is further enhanced by the efficiency of coupling the fluorescence back into the fibre, which also decreases with distance from the interface.

The geometry of optical fibres offers an advantage in terms of sensitivity in that the high number of internal reflections of a light ray propagating down an optical fibre are confined to two of the three dimensions instead of one, as for a planar waveguide. The total number of reflections (N) along a fibre is given by the formula;

$$N=(\frac{L}{2a})\cot\alpha \qquad (4.5)$$

where

L = fibre length

a = fibre core radius

α = incident wave angle to the normal to the interface

Since the interrogating light remains guided, no coupling optics are required in the sensor region and this allows considerable miniaturisation. Optical fibres also allow the transmission of light over long distances, facilitating remote sensing (Andrade *et al.*, 1985).

Some fluorescent molecules respond optically to changes in their environment, and this property can be exploited for the development of chemical sensors. The fluorescent intensity of fluorescein shows sensitivity to pH (Badini *et al.*, 1989) and certain derivatives of ruthenium have their fluorescence quenched by O_2 (Mac Craith *et al.*, 1993).

Evanescent wave sensors, based on non-fluorescent compounds have been reported. The wavelength of the propagating light is chosen so that it coincides with the absorbance wavelength of the analyte. The decrease in transmission of the propagated light (ATR), can be related to the concentration of the absorbing analyte. In 1992, Carlyon *et al.* reported a single mode fibre optic evanescent wave biosensor for the detection of a blue dye at 633 nm. The sensor was used for the determination of Penicillin G and was based on a colorimetric assay system.

4.6 Evanescent wave fibre optic immunosensors

Immunosensors require that the change induced by antibody-antigen binding is immediately convertible to an electrical signal that can be amplified, stored and displayed, so that as the primary bioreaction occurs, there is an immediate detectable signal output. Fluorophore-labelled antibodies (or antigens) can be used to detect analyte concentration, because the recognition event can be transduced indirectly, as a change in the fluorescence intensity, and detected via fibre optic evanescent wave technology. One of the first fluoroimmunosensors based on evanescent wave excitation and detection was developed by Sutherland *et al.* (1984).

Antibodies specific for the analyte of interest are immobilised on the exposed core silica of an optical fibre (Figure 4.3). The antibodies biospecifically bind analyte molecules, removing them from the bulk solution. Fluorophore-labelled antibodies with specificities for different epitopes on the analyte (antigen) surface, can simultaneously bind with the antigen to the antibodies bound to the fibre core surface. Fluorescent molecules, therefore, move into the evanescent 'sensing zone' of the fibre, in direct proportion to the analyte concentration, and are detected due to their interaction with the evanescent

wave. No separation step is required as with conventional plate immunoassays, since the evanescent wave only interacts with molecules within its penetration depth.

4.7 Immobilisation of antibodies to silica surfaces

The sensing surface, made up of antibodies immobilised on an optical substrate, e.g. an exposed core optical fibre, is fundamental to an immunosensor based on the evanescent wave technique. Methods for immobilisation include entrapment, adsorption, crosslinking, biological immobilisation (via Protein A or avidin-biotin) and covalent attachment and are discussed in detail in Taylor (1991).

The requirements for an efficient immunosensing surface include:

(1) high monolayer surface density of functionally active antibodies

(2) the absence of non-specific binding

(3) stability during storage and use

(4) linkage must be inert to dissociation conditions if sensor is to be reusable

(5) negligible denaturation of the immobilised antibody during the immobilisation procedure

The method of immobilisation must be simple (facilitating mass production), reproducible and should not require large amounts of antibody. Immobilisation via physical attraction i.e. adsorption, is not reliable because of problems concerning leaching and washing off the immunoglobulin during use. Jönsson *et al.* (1985), showed that covalently bound IgG has a superior stability compared to adsorbed IgG, when the pH was lowered or on incubation with detergents, urea or ethylene glycol. Adsorption followed by crosslinking with a bifunctional crosslinker, such as glutaraldehyde, has been shown to improve the stability of the adsorbed surface. However, protein-protein crosslinking generally results in some inactivation of the antibodies due to incorrect orientation and masking of the binding sites.

Directional orientation of antibodies with biological molecules, such as Protein A, which binds the Fc portion of the antibody, has shown increased activity per mole of protein immobilised. However, such reagents are extremely expensive and are prone to leaching (Alaire *et al.*, 1990).

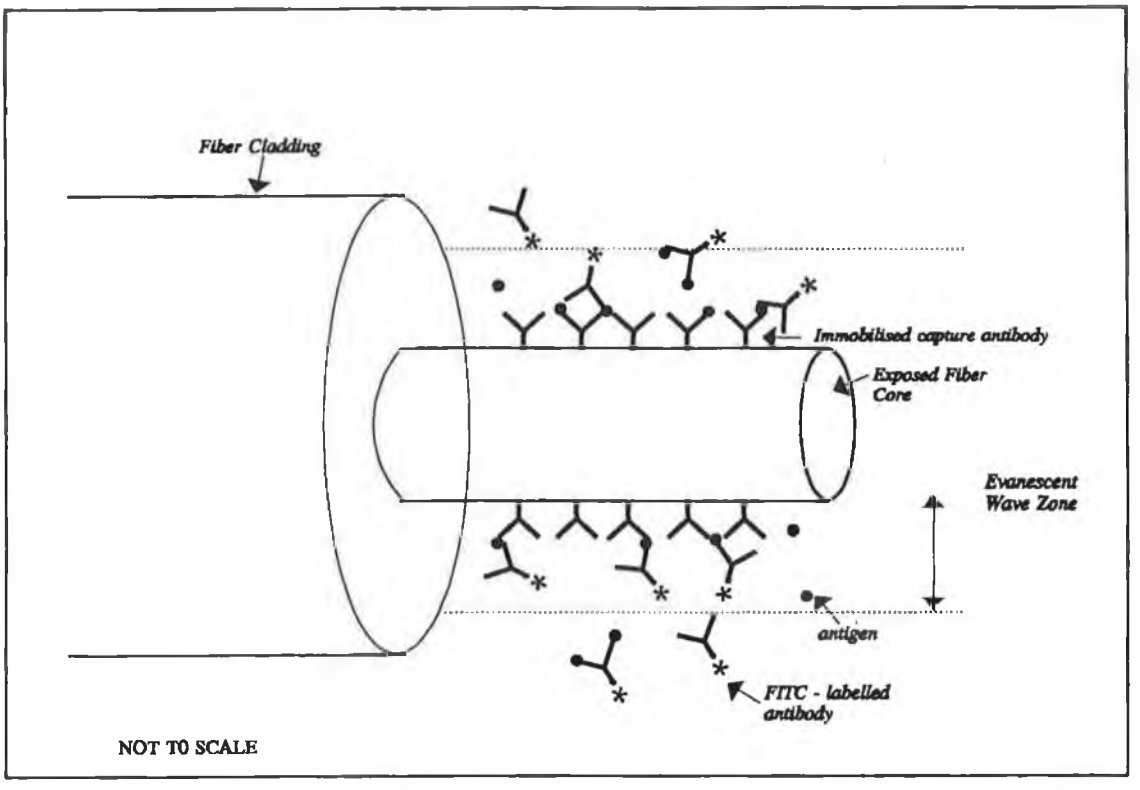


Figure 4.3: Evanescent wave fibre optic fluoroimmunosensing

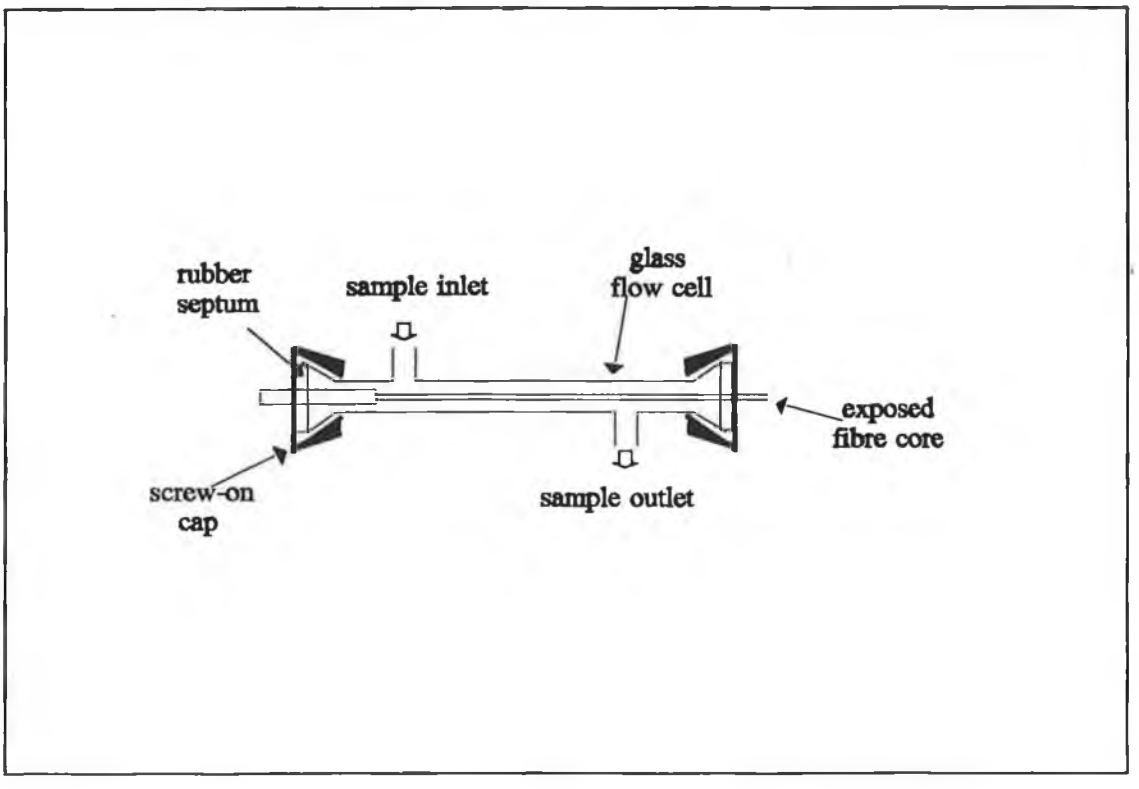


Figure 4.4: Fibre probe and flowcell apparatus for evanescent wave fluoroimmunosensing experiments

Several methods for covalent immobilisation of immunoglobulins onto silica surfaces have been reported. Surface derivatisation using silanes is a common way to impart a particular chemical reactivity to solid substrates which have hydroxyl groups on their surfaces, such as silica. Silane coupling reagents have one end which react with glass via one or more reactive groups and one end which gives the glass a new functionality. Initially developed methods utilised glutaraldehyde as a homobifunctional crosslinker, crosslinking the amino group of the immunoglobulin molecule to the silica substrate silanised with aminopropyltrimethoxysilane, APTS, (Weetall, 1976). As before, the use of homobifunctional crosslinkers suffers from multi-layer protein complex formation, thus, masking the activity of the antibody. Some silanes such as APTS are prone to hydrolysis and cyclisation (Cass, personal communication).

Brüning and Grobe (1992), synthesised several immobilisation reagents based on silanes with aldehyde functions. These compounds avoid the use of bifunctional crosslinkers, because the biomolecule reacts directly with the aldehyde groups, thus simplifying the procedure. Build up of polymer layers due to crosslinking of proteins, is also avoided. Zull *et al.* (1994), suggested that using long chain silane compounds for protein immobilisation results in more stable linkages as short chain silanes are subject to hydrolysis. They also suggested that exploiting the less common functional groups of the protein, such as the thiol groups of cysteine residues, will result in more reproducible site-directed coupling. Ahluwalia *et al.* (1992) and Baldini and Bracci (1993), compared several silanisation reagents under different conditions for IgG immobilisation.

The method used for antibody immobilisation in this thesis was that of Bhatia *et al.* (1989). A thiol terminal silane, MTS, was used to functionalise the glass surface. The thiol group reacts specificially with the maleiimide region of the heterobifunctional crosslinker, GMBS, leaving the succinimide residue of the GMBS available for protein attachment (Figure 4.5). This non-directional method of immobilisation was shown to retain the high antibody functionality. It was 3-5 times better than other investigators. No multilayer protein build-up occurs, due to the use of a heterobifunctional crosslinker.

A very new and novel approach to immobilising biological molecules on silica surfaces is via the sol-gel process. Porous glass coatings (sol-gels), on unclad fibres have been utilised to enhance the performance of evanescent wave sensors (Mac Craith, 1993). Initially, the sol-gel preparation methods were too harsh to allow immobilisation of organic molecules. However, more ambient preparation methods, described by Dunn *et*

al. (1991), facilitated the encapsulation of proteins such as enzymes (Shtelzer and Braun, 1994) and antibodies (Wang *et al.*, 1993) into sol-gel matrices while still retaining their biological activity. This technology is as yet in the early stages of development.

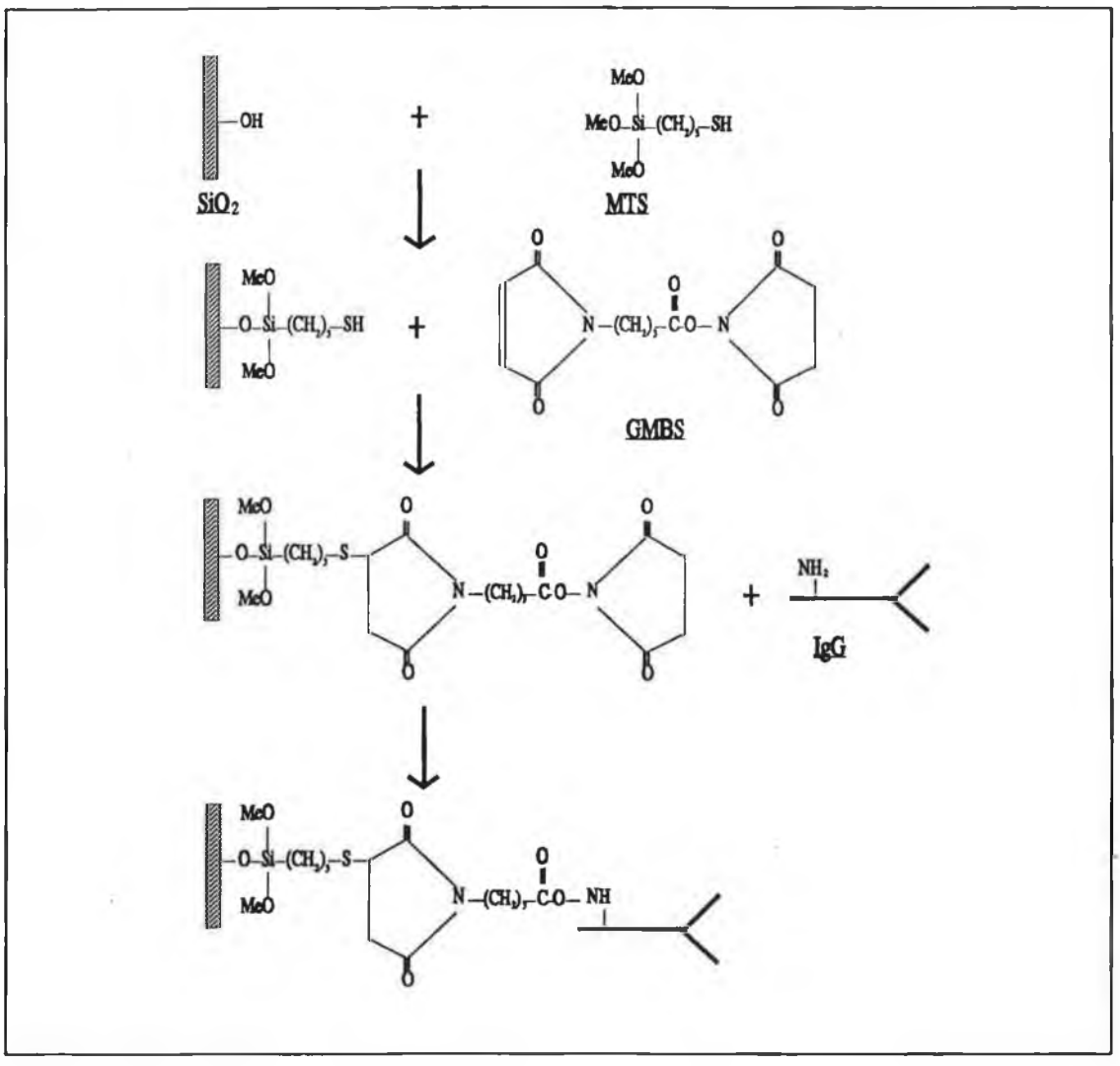


Figure 4.5: Immobilisation procedure for covalent attachment of antibodies to silica surfaces (Bhatia et al., 1989). The thiol terminal silane, mercaptopropyltrimethoxysilane (MTS), reacts with the acid-cleaned silica surface followed by the heterobifunctional crosslinker, N-γ-maleimidobutyryloxy succinimide ester (GMBS). The amino groups on the antibody then react with the succinimide ester group of the GMBS, forming stable amide bonds.

Results

4.8 Antibody immobilisation to exposed core optical fibres.

The procedure used for immobilising antibodies onto the exposed core of optical fibres, was that of Bhatia *et al.* (1989), as described in section 2.10.2. This method was chosen because it reported the ability to immobilise a high density of functional antibodies onto silica surfaces.

4.8.1 Optimisation of coating antibody concentration by ELISA techniques

The sensitivity of immunoassays is partially dependent on the amount of antibody immobilised onto the solid surface, in this case, the fibre core surface. In order to ensure that the maximum amount of antibody was immobilised to the substrate, without wastage of antibody, different coating antibody (rabbit anti-CHLDH antibody) concentrations were prepared (0, 0.5, 2.5, 5, 10, 40 and 80 µg/ml). Goat anti-mouse IgG (50 µg/ml), was immobilised on control fibres. Test tube ELISA's employing HRP-anti-rabbit IgG were performed on the fibres, as described in section 2.11.2.

From Figure 4.8.1, it can be seen that the absolute coating capacity of the fibres occurs for antibody coating concentrations of 40 µg/ml or greater. A coating antibody concentration of 50 µg/ml was used for all further immobilisations, to ensure maximum antibody immobilisation. The control fibres coated with goat antibodies, gave absorbance values similar to those not coated with antibody (0 µg/ml). Since it is generally accepted that less than 10% of the antibody in solution actually binds to the fibres, the antibody solution was reused 2-3 times. Theoretically, it could be reused more times. However, since no sodium azide (NaN_3) preservative could be added, as it inhibits the coupling reaction between IgG and ester groups, diluted antibody solutions were discarded after 2 weeks.

4.8.2 Reproducibility of antibody immobilisation using ELISA techniques

In order to ensure that the sensor system gave reproducible results, it was necessary to ensure that the same amount of antibody was immobilised onto each fibre in a batch, at a given coating antibody concentration. A different fibre was used for each sample reading in the evanescent wave immunosensor system, and so fibre-to-fibre variation would result in inter-assay error. Eight fibres were incubated with each coating antibody (0 - 80 µg/ml rabbit anti-CHLDH antibody) concentration and probed with HRP-anti-

rabbit IgG, as outlined in section 2.11.2. Control fibres (50 µg/ml goat anti-mouse IgG) were also included. The absorbance at 405 nm was recorded for each fibre, and the %CV for each antibody concentration determined, where n=8.

From the graph (Figure 4.8.2), the immobilisation method was shown to give good 'inter-batch' reproducibility. The data for concentrations 60 and 80 µg/ml showed some overlap as they are on the saturation part of the curve (see Figure 4.8.1). The %CV for all sets of data points was <4%, indicating reproducible immobilisation of antibodies.

4.9 Fibre blocking to reduce non-specific binding

Another factor which must be taken into account is non-specific binding. Non-specific binding occurs due to interactions of the antigen or second labelled antibody with the fibre core surface, rather than the immobilised antibody, and can be dependent to some degree on the immobilisation chemistry used. Blocking all available binding sites on the fibre core surface helps reduce non-specific binding. Bovine serum albumin (1%, w/v) and gelatin (1%, w/v) were tested as blocking agents in fibre ELISA systems, and it was concluded that bovine serum albumin (1%, w/v) effectively blocks non-specific binding to less that 8% (data not shown).

4.10 Working range of anti-CHLDH antibody coated fibres by ELISA techniques

The sensitivity range (i.e. the range of antigen concentrations that can be detected), of the evanescent wave immunosensor will partially depend on the amount of immobilised active antibody on the fibre surface. The approximate sensitivity range was determined by fibre ELISAs, as outlined in section 2.11.1. It was determined to be 0.125 - 1.000 µg/ml, for the two-step immunoassay (Figure 4.10.1).

A one-step immunoassay was also performed on the fibres (Figure 4.10.2), as outlined in section 2.11.1, and a wider linear range for the ELISA was obtained, 0.05 - 1.00 µg/ml.

Figure 4.8.1

Optimal coating concentration for immobilisation of affinity-purified anti-CHLDH antibodies to the exposed core fibre surface, using the method of Bhatia *et al.* (1989). Different concentrations (0-80 µg/ml) of coating antibody were immobilised to the fibre surface and the optimal coating concentration determined by ELISA, as outlined in section 2.11.2. Above 40 µg/ml, the fibre is saturated with antibody and increasing the coating antibody concentration has no effect.

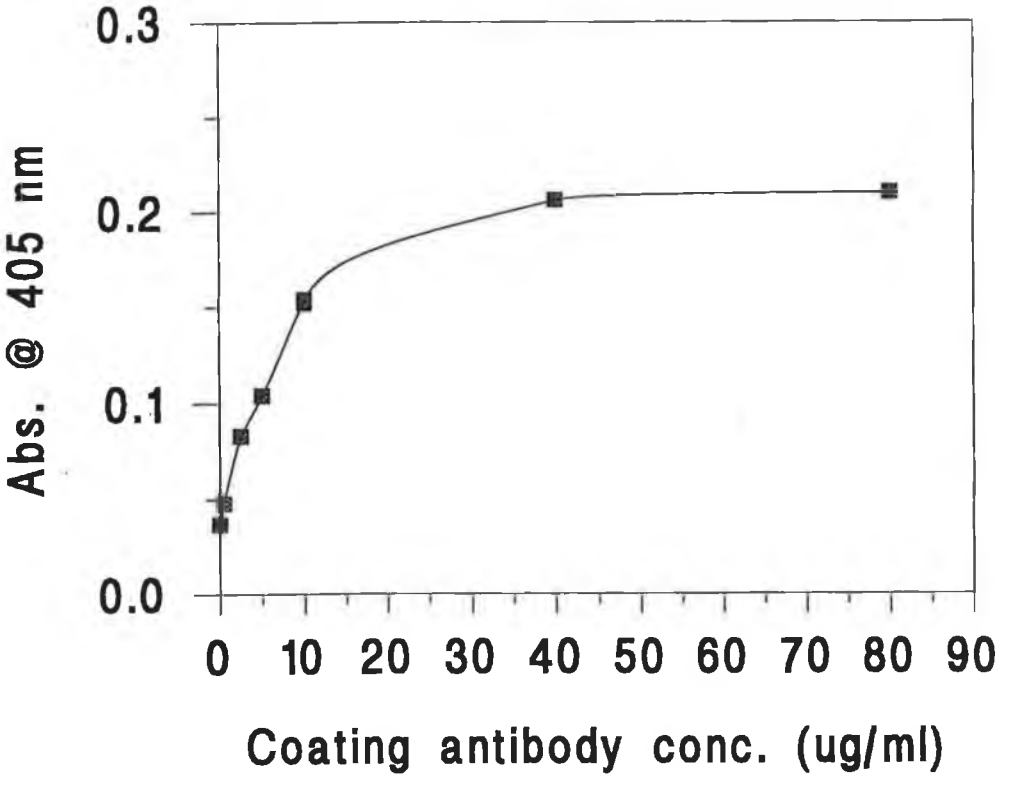


Figure 4.8.2

Inter-batch reproducibility of the immobilisation of affinity purified anti-CHLDH antibodies to exposed core fibre surface using the method of Bhatia *et al.* (1989). Fibres (n=8) were coated with different concentrations of the antibodies (0 (○), 5(♦), 20(□), 30(■), 60(x) and 80(+) µg/ml), and the reproducibility assessed qualitatively by fibre ELISA as outlined in section 2.11.2. The coefficient of variation was determined to be < 4%, indicating good inter-batch reproducibility for the immobilisation procedure.

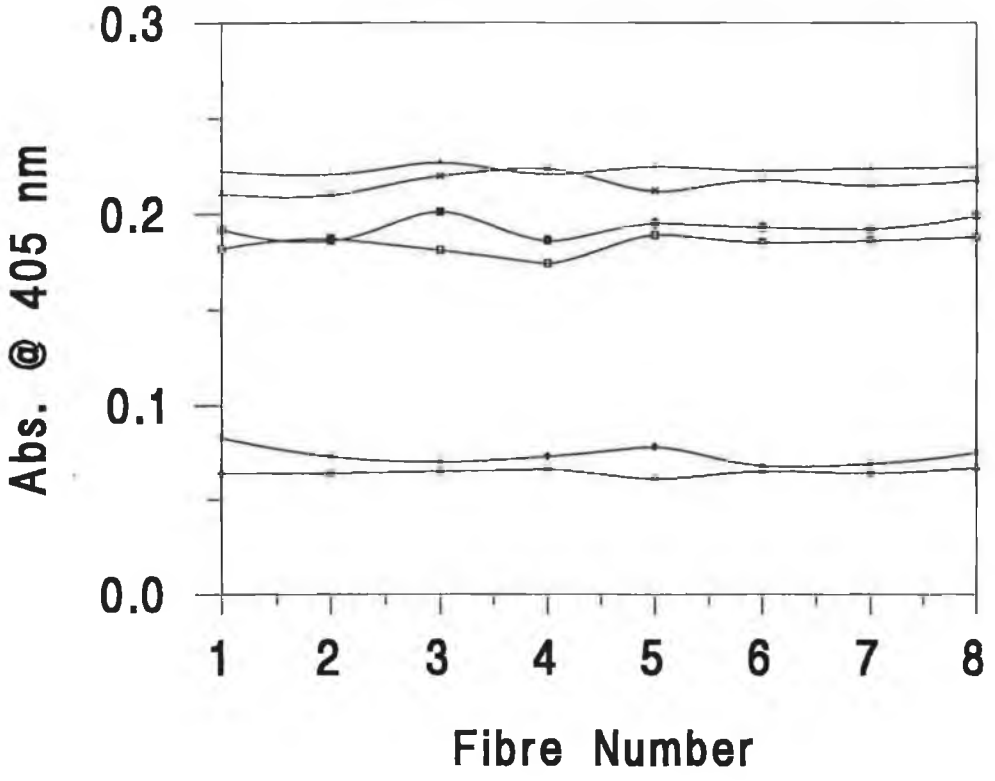


Figure 4.10.1

Sensitivity range of anti-CHLDH antibody-coated fibres as determined using a two-step fibre ELISA (section 2.11.1). The fibre sensitivity range was determined to be linear between 0.05-1.00 µg/ml. All sample estimations were performed in triplicate, and the HRP-anti-CHLDH was diluted to 1:4000.

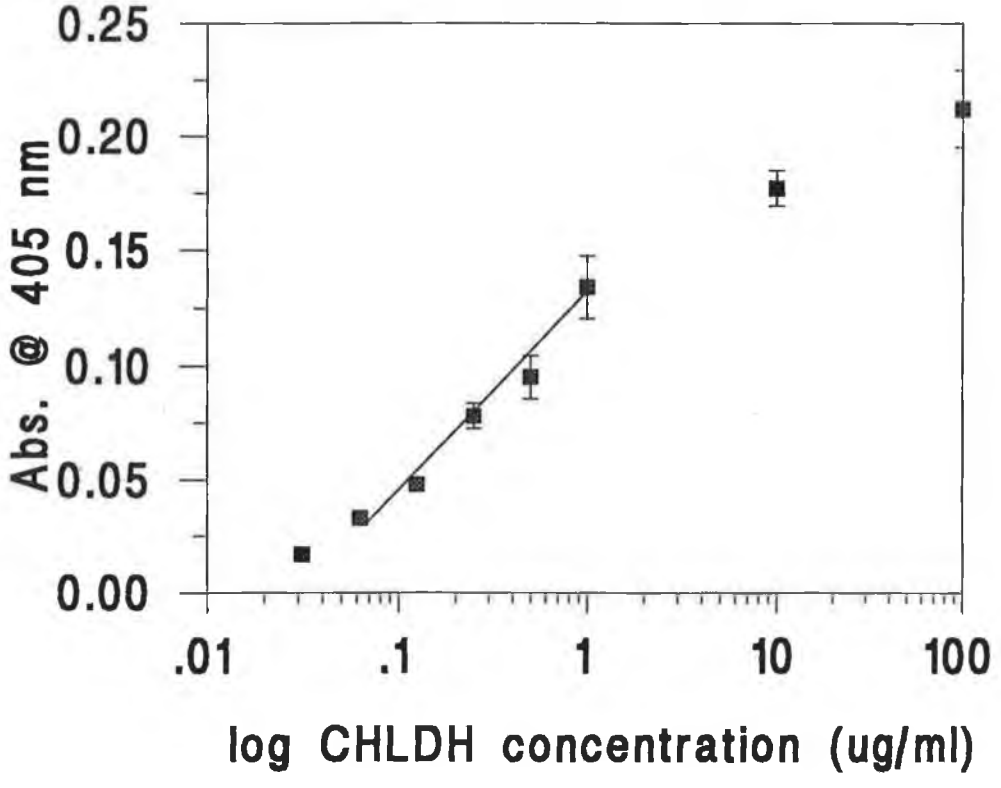
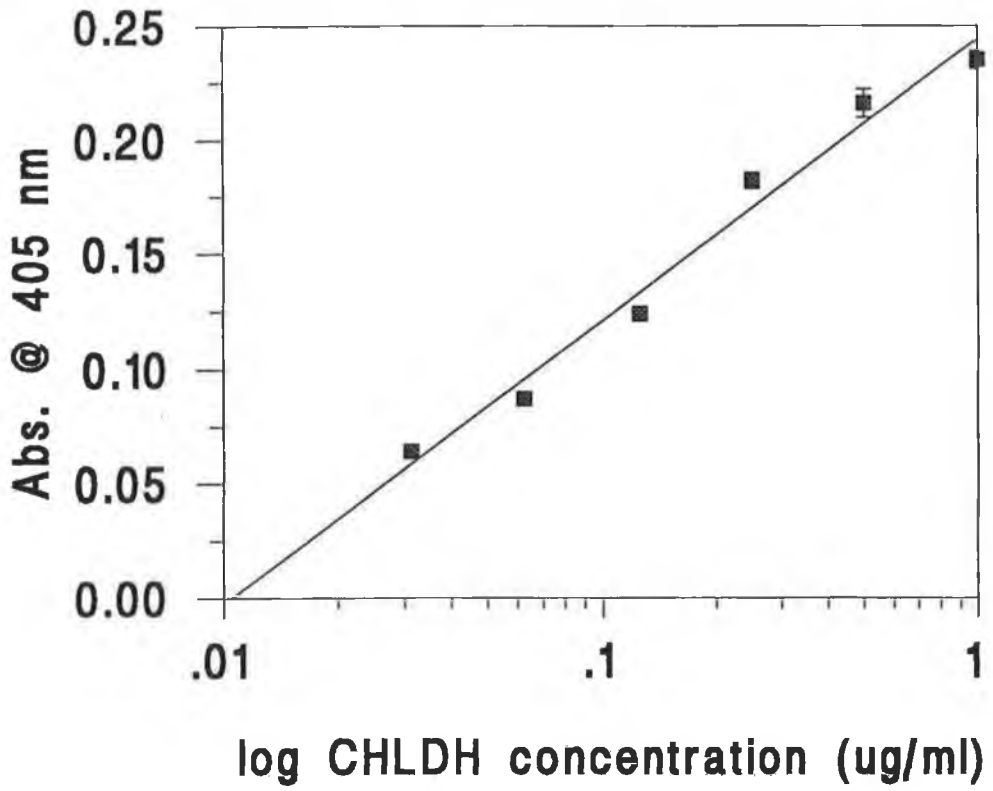


Figure 4.10.2

Linear working range of anti-CHLDH antibody-coated fibres as determined using a one-step fibre ELISA (section 2.11.1). The fibre working range was determined to be 0.05-1.00 µg/ml (r=0.990). All sample estimations were performed in triplicate, and the HRP-anti-CHLDH antibody was diluted to 1:4000.



4.11 Evanescent wave immunosensing

The system used to carry out this work is described in section 2.12.1 and Figure 2.3. The argon-ion laser has a 488 nm line and so is suitable for excitation of FITC which absorbs strongly at 488 nm for fluorescence emission at 520 nm (Figure 4.2).

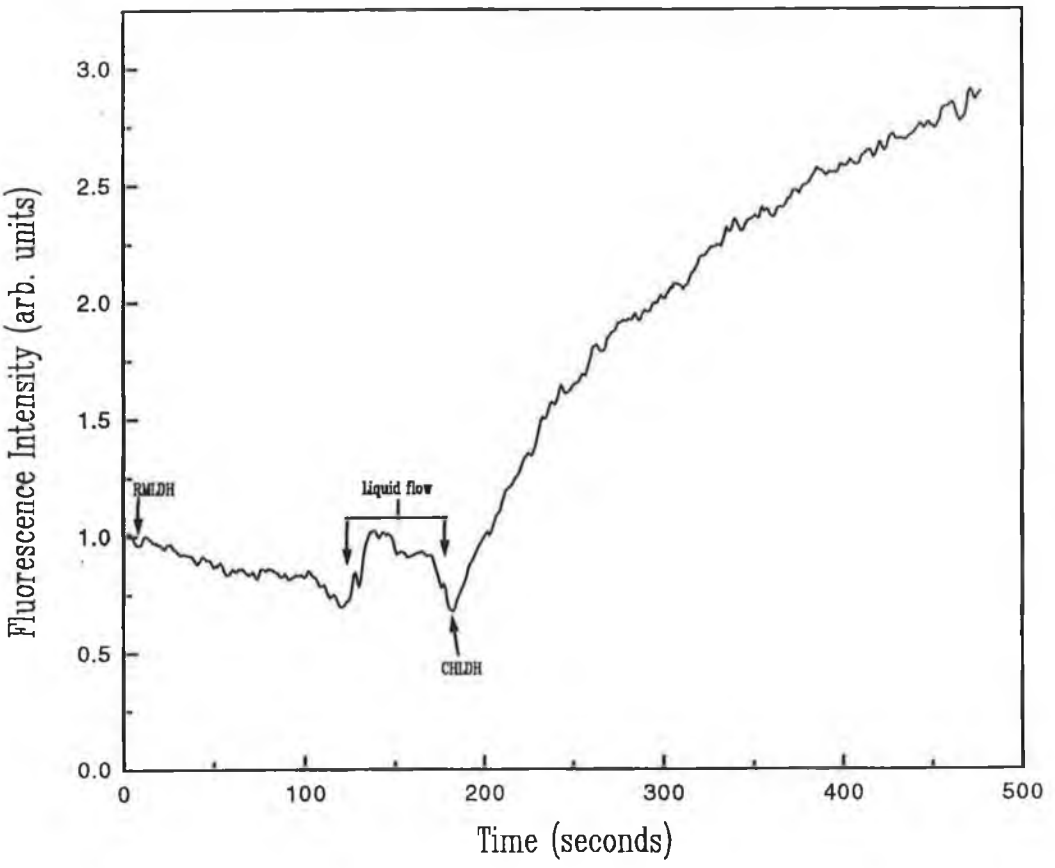
A glass flow cell with both ends threaded, to facilitate screw-on caps, was designed by the author and manufactured by AGB Scientific, Ireland (Figure 4.4). It is essential that the fibre is held firmly in place in the flow cell, so that it does not move out of position when liquids are being pumped through the flow cell. The fibre is threaded through rubber septa at either end of the cell, and held along the central axis of the flow cell, using the screw-on caps. The flow cell was filled with BSA (1%, w/v), to obtain a baseline signal, prior to adding the sample. Samples were passed through the flow cell using a peristaltic pump in such a way that no air bubbles entered the sample flow stream. A new fibre was used for each sample (except for the regeneration experiments). The flow cell was mounted in an X-Y-Z micropositioner to enable adjustment of the fibre launching position.

4.11.1 Specificity of fibre probe

The fibre was aligned in the optical system (Figure 2.3), and a baseline recorded after filling the flow chamber with 1% (w/v) BSA. D.C. background signals, due to excitation light breaking through the emission filters and back reflections from the proximal end of the fibre, were nulled by using the 'offset' feature of the lock-in amplifier. Non-specific antigen, 1 µg/ml rabbit muscle LDH (RMLDH), was premixed with 20 µg/ml FITC-labelled anti-CHLDH antibody prepared in 1% (w/v) BSA, and passed over the fibre using the peristaltic pump at a flow rate of 5 ml/min. No increase in signal over the baseline was recorded, indicating that there was no non-specific binding of antigen and antibody (Figure 4.11.1). A slight decrease in signal was actually observed. When the fibre was exposed to excitation light continuously, the innate fibre fluorescence slowly decays and leads to a changing background signal. For quantitation experiments the baseline was allowed to stabilise before measurements were taken.

Figure 4.11.1

Specificity of fibre probe for CHLDH. Rabbit muscle LDH, RMLDH (1 µg/ml), premixed with 20 µg/ml FITC-anti-CHLDH antibody and injected into the flow cell containing the fibre probe. No increase in fluorescence signal by evanescent wave sensing was observed. CHLDH (1µg/ml), premixed with 20 µg/ml FITC-anti-CHLDH antibody was then passed over the fibre probe. An increase in the fluorescent signal was observed due to the specific binding of CHLDH/FITC-anti-CHLDH antibody immunocomplex with the fibre-immobilised antibody.



A sample consisting of 1 µg/ml specific antigen, chicken heart LDH (CHLDH) premixed with 20 µg/ml FITC-labelled anti-CHLDH antibody prepared in 1% (w/v) BSA, was then passed over the fibre. An immediate signal increase was observed. This fluorescence intensity increase was attributed to the specific binding of the FITC-anti-CHLDH/CHLDH immunocomplex to the fibre core immobilised anti-CHLDH antibody, and interaction of the FITC with the evanescent wave. This experiment shows that the increase in fluorescence signal is due to the immunoreaction and not non-specific binding.

The small increase in signal observed between the non-specific and specific antigen samples, was due to vibrations in the fibre during liquid flow, and is not a fluorescence signal.

4.11.2 Regeneration of fibre probe

Many antibody-antigen reactions have high affinity constants of the order of 10^{10} - 10^{12} M^{-1}, which confer high sensitivity on immunoassays. The price to be paid, however, is irreversibility, and hence most immunosensing systems are considered single use with irreversible binding. However, if the fibre immunosensing surface can be regenerated for reuse, several measurements may be made with one fibre.

The fibre probe was aligned in the optical system and a one-step immunoassay (2 µg/ml CHLDH premixed with 20 µg/ml FITC-anti-CHLDH antibody) performed on the fibre, 7 times, with regeneration in between each sample. Glycine-HCl (0.1M), pH 2.5, for 1.5 min was used for the regeneration of the fibre probe, as this buffer was found to successfully break the antibody-antigen complex in question, when purifying the antibody by affinity chromatography (section 2.4.2.2). A typical signal curve with regeneration is shown in Figure 4.11.2.1. The fluorescence signal generated by the immunoreaction, was monitored for approximately 2 minutes, and the initial linear slope (over a time period of 0.5 min) of the curve determined using linear regression.

The initial slope of the immunoreaction curve is plotted against the number of regenerations, in Figure 4.11.2.2. For the 7 regenerations carried out, is does not appear that the dissociating conditions used, had any adverse effect on the antigen binding ability of the immobilised antibody. The %CV of the 7 samples was 5.3%. No further regenerations were carried out, as it was impossible to keep air bubbles out of the flow cell, without removing the fibre and flow-cell apparatus from the X-Y-Z micropositioner,

filling the flow cell and realigning the fibre. Realignment has been shown to induce some variability (see section 4.11.3).

It was observed that after the dissociating solution was pumped out of the flow cell and baseline solution (1% (w/v), BSA), pumped in, the new baseline signal was slightly higher than the initial baseline signal, indicating that not all of the immunocomplexes had been removed from the surface. With further regenerations, the kinetics of the immunoassay would be changed, due to their being progressively less solid-phase immobilised antibody available for binding (Place *et al.*, 1991). For this reason, it was decided to use a new fibre for each new sample.

4.11.3 Realignment reproducibility of fibre probe

For every new sample assayed, a new fibre probe had to be aligned in the optical system. This proved difficult because unlike other evanescent wave sensors, such as those based on the quenching of fluorescence, there was no initial fluorescence signal (O'Keeffe *et al.*, 1995). Fluorescence allows easier optical alignment, as the light spot can be visually focused on the detector, and the monitored signal maximised. For this system, the fluorescence was generated after optical alignment, due to the fluorescently labelled antibodies in the immunoreaction. Exact alignment is crucial, to ensure all the angles of the fibre are filled.

The fibre probe was realigned in the system each time using the X-Y-Z micropositioner and judged by eye, using the light spot emerging from the distal end of the fibre as a guide. For evanescent wave sensing at the proximal end of the fibre, there is normally no need to polish the distal end of the fibre. However, in this case the distal end of the fibre was polished, because it allowed a circular light spot to be focused on the back of the hand. When this light spot was symmetrical and perfectly circular, the fibre was considered aligned.

A one-step immunoassay was performed (2 µg/ml CHLDH premixed with 20 µg/ml FITC-anti-CHLDH antibody), eight times using a new fibre probe for each reading. The initial signal was monitored and the slope (mV/sec) determined. The %CV for the eight probes was determined to be 14.4% (Figure 4.11.3). This error was attributed to the difficulty in aligning the fibre accurately. There is also the possibility of other variables in the system, such as fibre-to-fibre variation in optical transmission properties and polishing defects, although care was taken to reduce these.

Figure 4.11.2.1

FITC-anti-CHLDH antibody/CHLDH immunocomplex binding to immobilised anti-CHLDH antibody (i.e. one-step assay) and detection by evanescent wave sensing, followed by regeneration of the fibre probe surface with 0.1M glycine, pH 2.5. The rapid drop in fluorescence after addition of the acidic solution is due to the quenching of the fluorescence of fluorescein at low pH and dissociation of some of the immunocomplexes from the fibre probe. On filling the flow-cell again with baseline solution (1% (w/v) BSA), the fluorescence intensity was higher than that of the original baseline solution, indicating that not all of the immunocomplexes had been dissociated from the fibre surface.

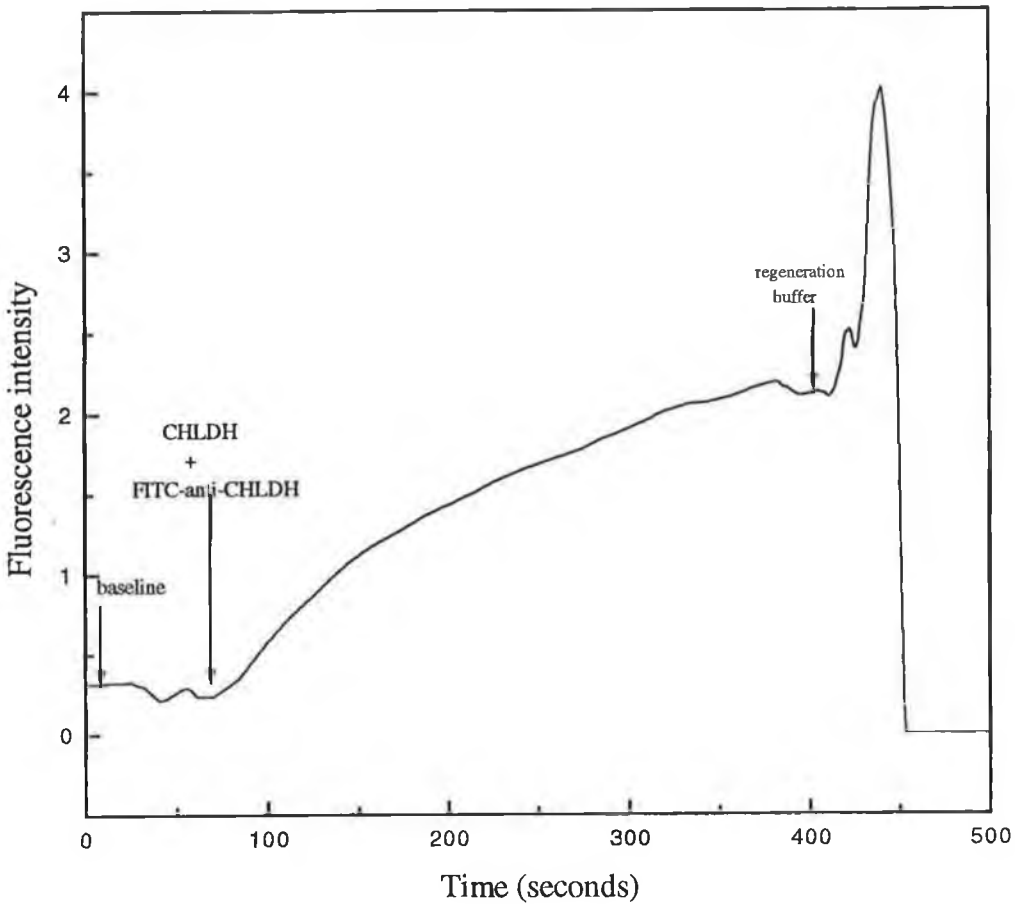


Figure 4.11.2.2

Effect of repeated regenerations with 0.1M glycine, pH 2.5 on the binding ability of fibre probe. The effect of repeated regeneration on the signal measured by evanescent wave sensing for a one-step immunoassay (2 µg/ml CHLDH premixed with 20 µg/ml FITC-anti-CHLDH antibody) was determined (n=7). The sensitivity of the optical system was set at 100mV. The same fibre was used for all experiments and the fibre probe was not realigned between samples. The coefficient of variation for n=7 was 5.3%.

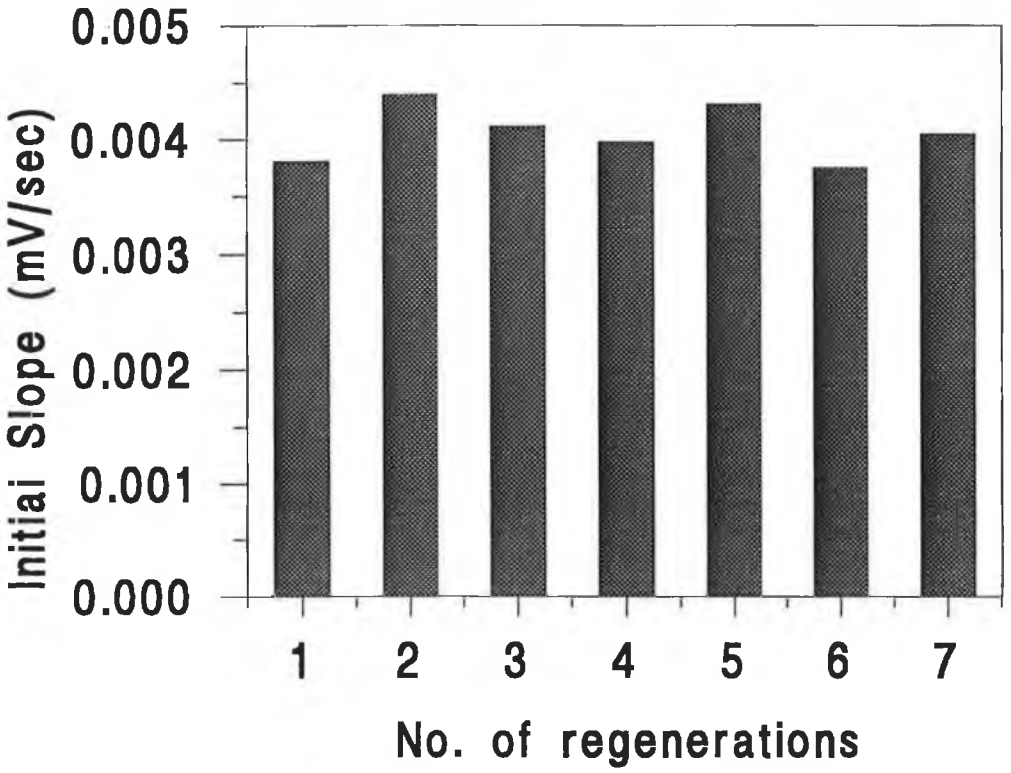
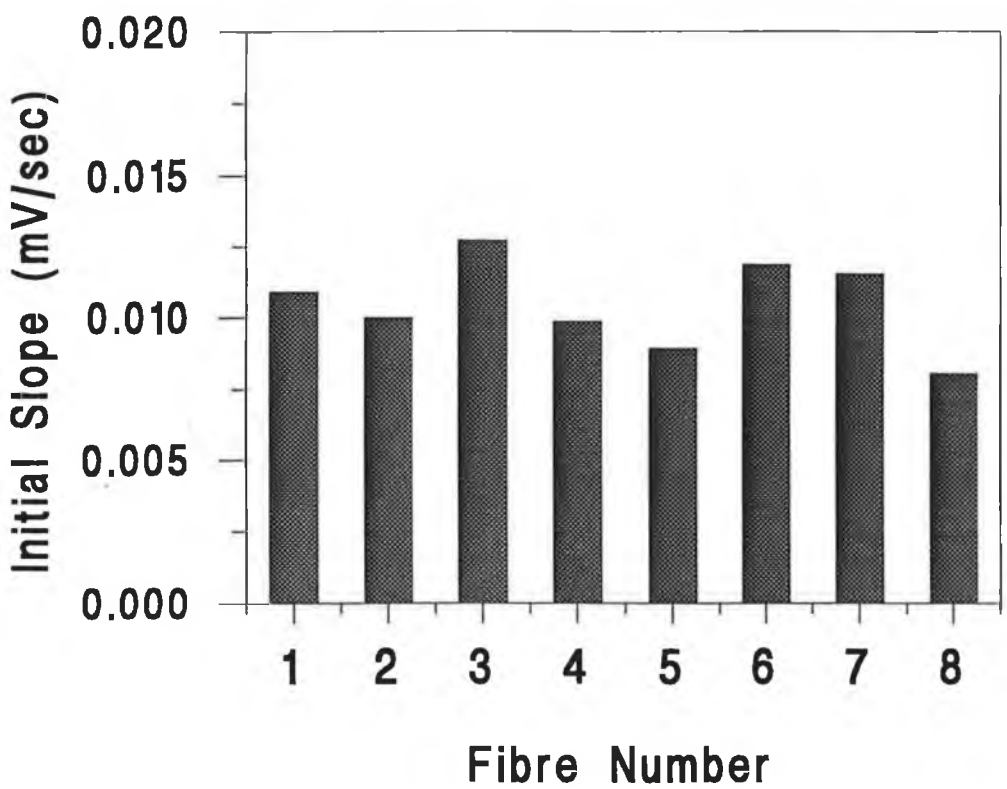


Figure 4.11.3

Effect of fibre realignment on the reproducibility of the signal measured by evanescent wave sensing for a one-step immunoassay (2 µg/ml CHLDH premixed with 20 µg/ml FITC-anti-CHLDH antibody). The sensitivity of the optical system was set at 100mV. A new fibre was used for each sample concentration (n=8), and the coefficient of variation was 14.4%, indicating that there is some variability in realigning the fibre probes.



4.11.4 Kinetic response of fibre-optic immunoassay

The slope of the signal response curve, which corresponds to the initial rise of the number of labelled antigen molecules (FITC-CHLDH) on the fibre surface, should be strictly proportional to the concentration of antigen in solution, for a diffusion-limited process (Feldman and Uzgiris, 1993). Antigen (FITC-CHLDH) was prepared at concentrations ranging from 0.125-5.00 µg/ml (1.12-35.7 nM), in 1% (w/v) BSA and injected into the flow cell. The increase in fluorescence intensity was recorded and the initial slope of the binding curve determined. The results are plotted in Figure 4.11.4. The results show that there is an excellent linear dependence for all data points except for the high concentration, 5 µg/ml. The reason for this is not clear. It is possible that at high concentrations of antigen, convectional effects and not diffusion through the 'dead layer', are the limiting factors in the mass transport process to the surface.

4.11.5 Two-step and one-step immunoassays

A two-step immunoassay was performed on the fibres as described in section 2.12.2.1. The fibres were incubated with CHLDH (0.03 - 10.0 µg/ml) for 15 mins, followed by 20 µg/ml FITC-anti-CHLDH antibody. The fluorescence signal was recorded for 4.5 min and the initial slope (over 0.5 min), calculated by linear regression (4.11.5.1). The sensitivity of the optical system was set at 300 mV, with the 'expand X10' function turned off. The linear range for the two-step assay was from between approximately 0.1 - 10.0 µg/ml (r=0.995), as shown in Figure 4.11.5.2. This is similar to results obtained with the fibre ELISA (Figure 4.10.1).

A one-step assay was also performed, where the antigen, CHLDH (0.03 - 10.0 µg/ml), was premixed with 20 µg/ml FITC-anti-CHLDH antibody(section 2.12.2.2), and injected into the flow cell immediately. The initial binding slope was calculated as before. The sensitivity of the optical system was 100mV, with the 'expand X10' function turned on. The results are shown in Figure 4.11.5.3. The linear range for the one-step assay was approximately 0.156 - 5.0 µg/ml (r=0.990), again giving similar results to the one-step fibre ELISA (Figure 4.10.2).

It is clear from Figures 4.11.5.2 and 4.11.5.3, that the two-step assay is more sensitive than the one-step assay. The optical system sensitivity was set 30-fold less for the two-step assay compared to the one-step assay, and yet the initial slope was 3-4 times higher for the two-step assay.

Figure 4.11.4

Kinetic response of fibre probe using FITC-CHLDH, showing pseudo-first order reaction kinetics. The slope of the signal response curve is strictly proportional to the concentration of antigen in solution, for a diffusion-limited process. There is a deviation from linearity at high antigen concentrations.

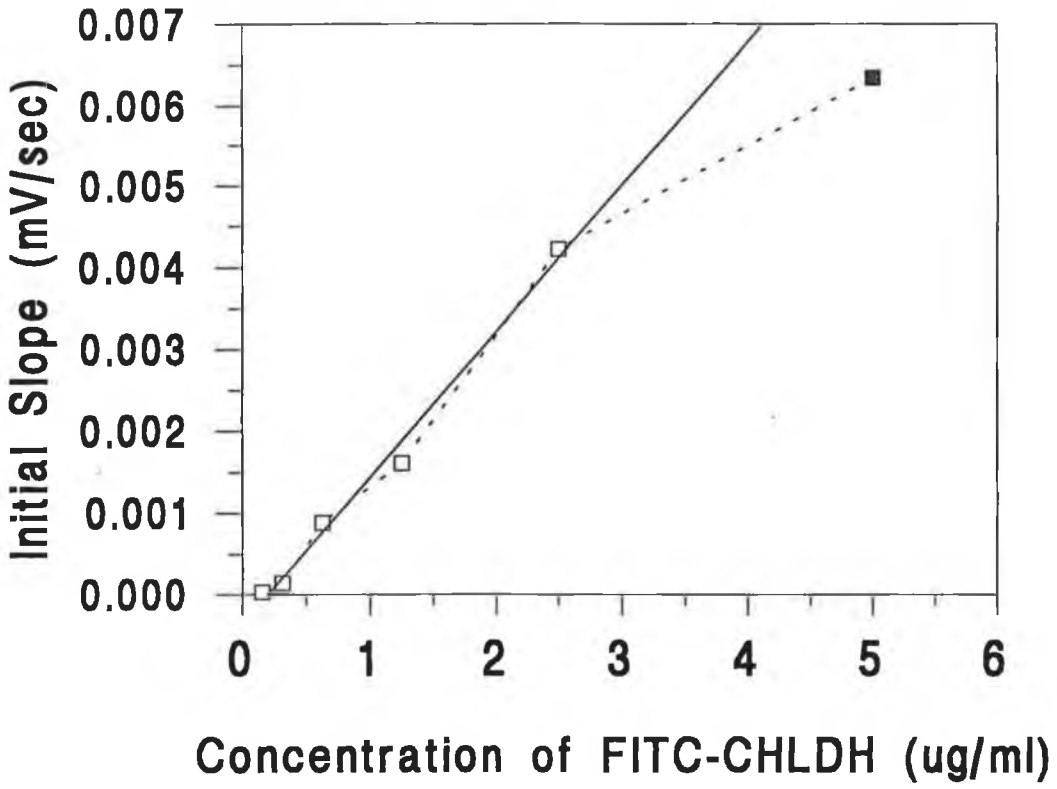


Figure 4.11.5.1

Typical one-step or two-step binding curve for evanescent wave immunosensing experiments. The rate of binding follows pseudo-first order reaction kinetics and, hence, linear regression was carried out on the initial part of the binding curve, which is directly proportional to the antigen concentration. The sample was injected into the flow cell at the point marked A, however the slope was determined from B-C. The first part of the curve was ignored (A-B), to eliminate any background fluorescence effects and fluorescence due to non-specific binding.

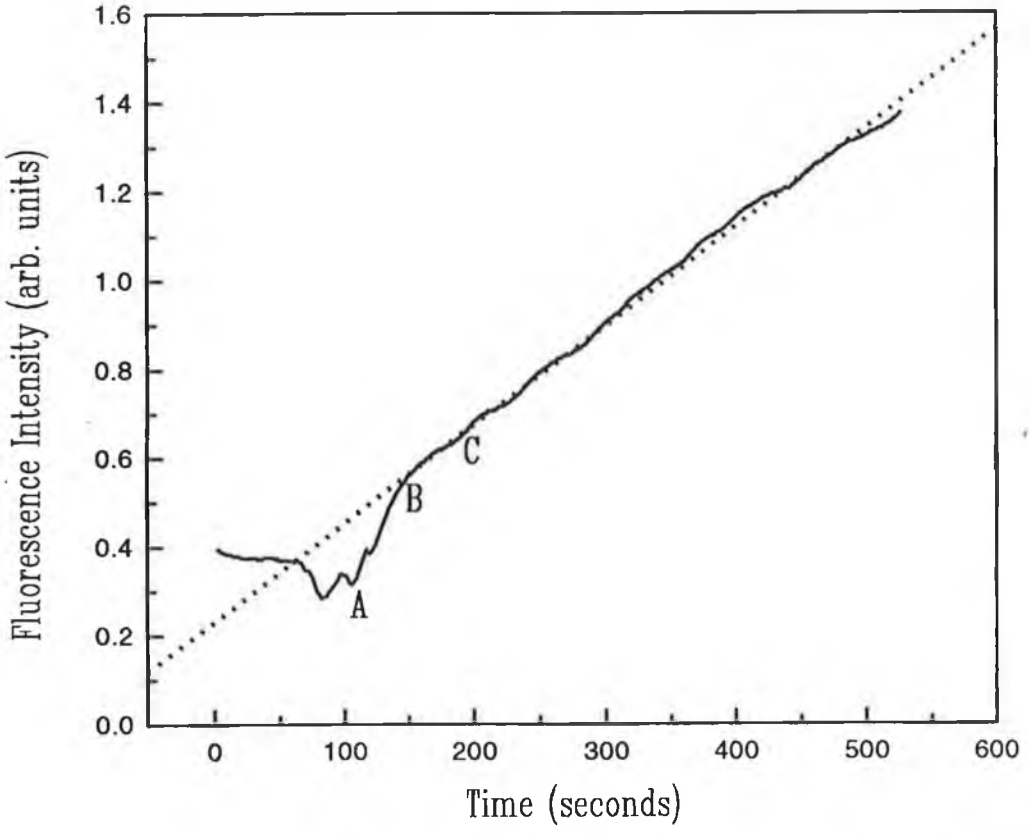


Figure 4.11.5.2

Standard curve for two-step immunoassay for CHLDH using evanescent wave immunosensing. The slope of the binding curve was determined by linear regression on the initial part of the curve, over 1 min. The linear range is between 0.1 - 10.0 µg/ml (r=0.995). The sensitivity of the optical system was set at 300mV, and the 'expand x10' function was off.

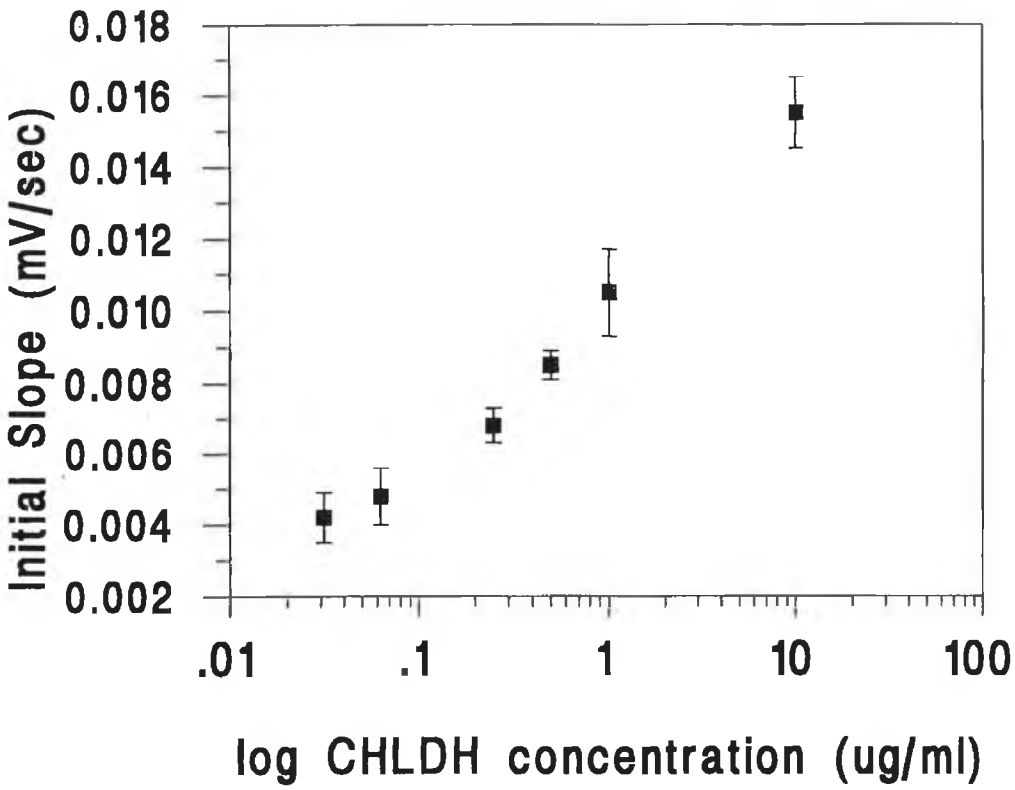
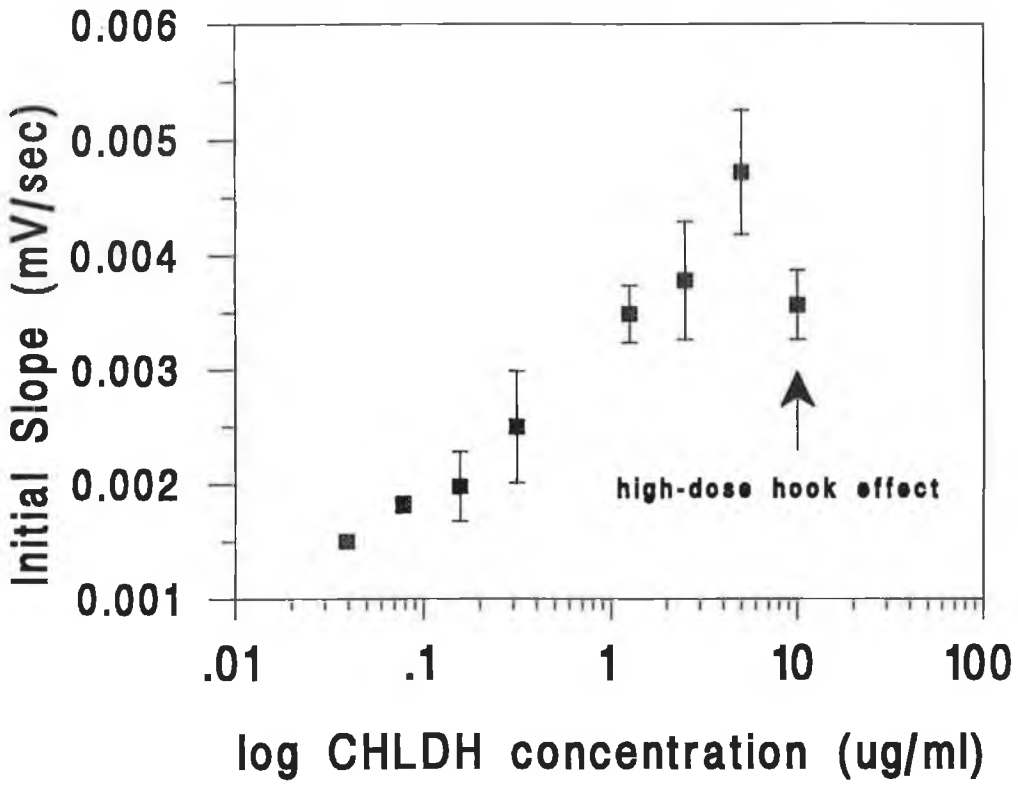


Figure 4.11.5.3

Standard curve for one-step immunoassay for CHLDH using evanescent wave immunosensing. The slope of the binding curve was determined by linear regression on the initial part of the curve, over 1 min. The linear range is between 0.156 - 5.0 µg/ml (r=0.990). A 'high-dose hook effect' for high CHLDH concentrations has occurred because the FITC-labelled anti-CHLDH antibody was no longer in excess. The sensitivity of the optical system was set at 100mV and the 'expand x10' function was on.



4.11.6 Competitive immunoassay

A competitive one-step immunoassay was performed for CHLDH concentrations ranging from 0.156 - 10.0 µg/ml. An excess of FITC-labelled CHLDH (~20 µg/ml), and standard CHLDH competed for the limited number of immobilised antibody binding sites. The signal was inversely proportional to the concentration of unlabelled CHLDH, as the more unlabelled CHLDH in the sample, the less sites available for the labelled CHLDH to bind.

The initial slope of the signal was calculated and the results are shown in Figure 4.11.6. The graph is linear for concentrations ranging from 1.0 -10.0 µg/ml (r=0.992).

4.11.7 Effect of sample viscosity on signal accumulation

All the samples assayed by the evanescent wave immunosensor were prepared in 1% (w/v) BSA. Serum has a viscosity similar to 8% (w/v) BSA. Sample matrices; 0.15M PBS, pH 7.4, 1% (w/v) BSA, 3% (w/v) BSA, 8% (w/v) BSA and a 1:2 dilution of normal human serum, were prepared in 0.15M PBS, and a one-step immunoassay performed (2 µg/ml CHLDH + 20 µg/ml FITC-anti-CHLDH antibody), in duplicate for each sample matrix. The aim of this experiment was to check whether the viscosity of the sample matrix effects the rate of accumulation of fluorescence intensity signal. The results are shown in Figure 4.11.7. It was expected that for higher viscosity solutions, the rate of signal accumulation would be less than for lower viscosity solutions. The opposite result was found. The reason for this is unclear, but may be an effect due to a change in the numerical aperture of the sensor, resulting in more efficient coupling of fluorescence back into the fibre, rather than a viscosity effect.

Figure 4.11.6

Standard curve for competitive immunoassay for CHLDH using evanescent wave immunosensing. The slope of the binding curve was determined by linear regression on the initial part of the curve, over 1 min. The linear range is between 1.0 - 10.0 µg/ml (r=0.992). The sensitivity of the optical system was set at 100mV and the 'expand x10' function was on.

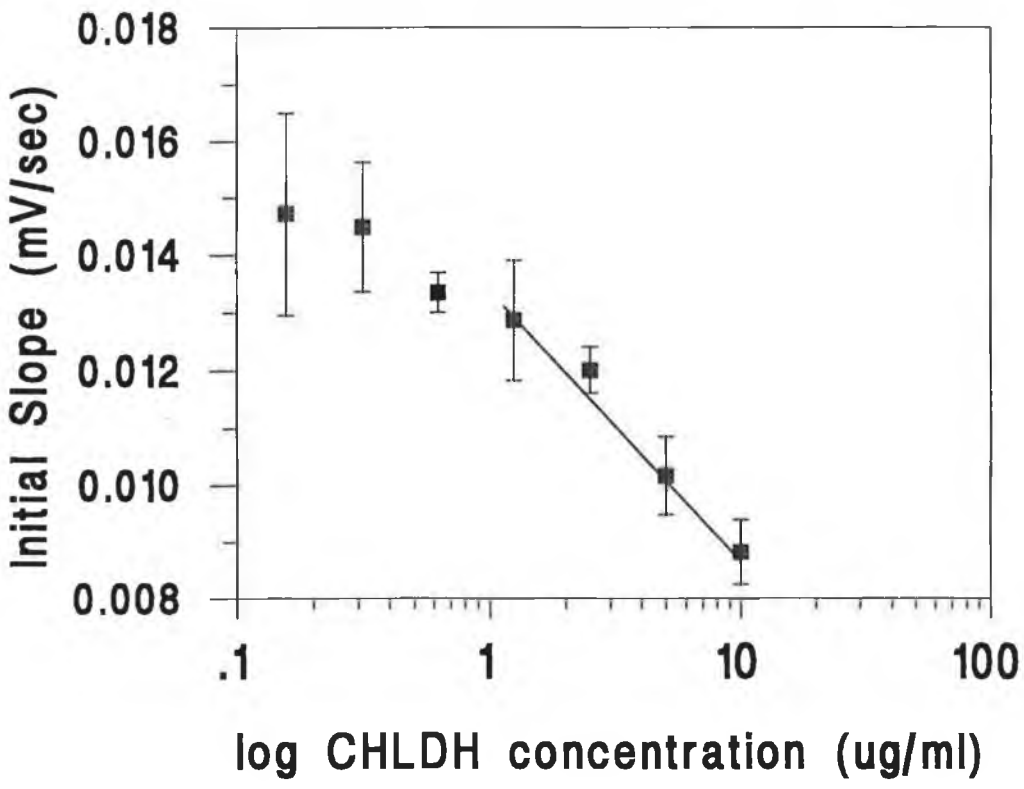
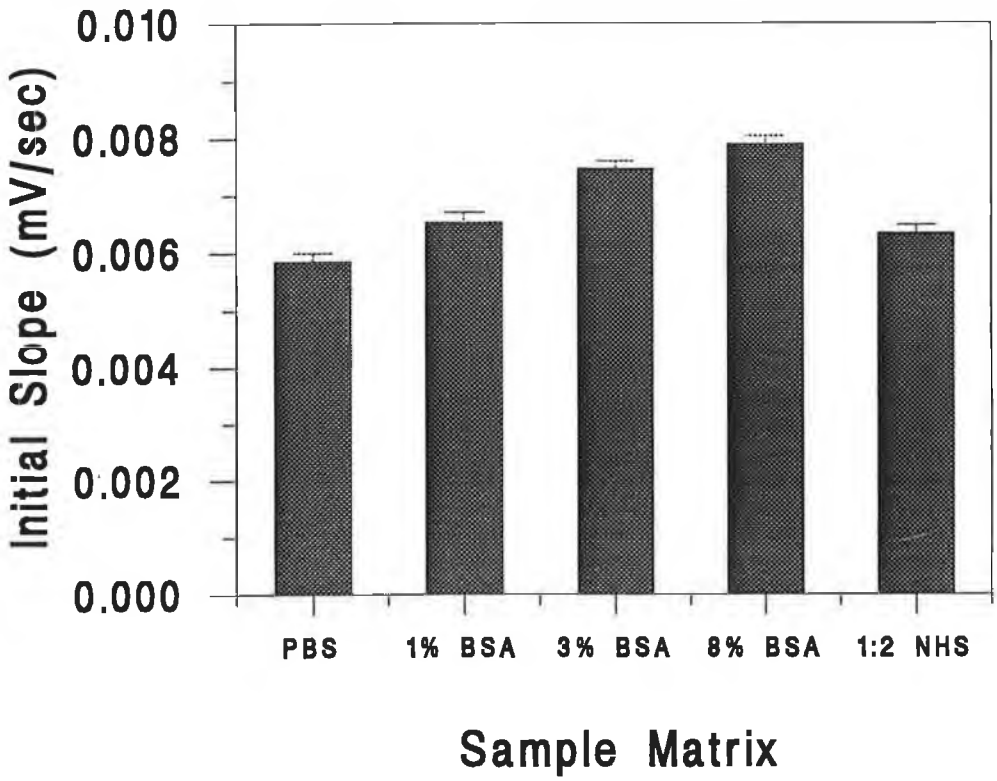


Figure 4.11.7

Effect of sample matrix viscosity on signal accumulation. A one-step immunoassay (2 µg/ml CHLDH premixed with 20 µg/ml FITC-anti-CHLDH antibody) was performed in duplicate in matrices of different viscosities and the effect on the rate of signal accumulation determined by linear regression on the initial part of the binding curve.



4.12 Determination of CHLDH concentration using the BIAcore™

The BIAcore™ uses the optical phenomenon of surface plasmon resonance (SPR), to measure the surface concentration of analyte biospecifically bound to the sensor chip surface. The principle of SPR is described in section 4.7.

Anti-CHLDH antibodies were covalently immobilised to the sensor chip surface as outlined in section 2.13.1. A typical immobilisation sensorgram is shown in Figure 4.12.1. Direct (no second antibody), one-step and two-step immunoassays were performed on the sensor chip surface coated with anti-CHLDH antibody. A typical antibody-antigen sensorgram curve is shown in Figure 4.12.2. Different combinations of regeneration solutions were tested (0-100mM HCl; 0-100mM NaOH; 0.1M glycine, pH 2.5), to evaluate how the immunosensing surface could be most effectively regenerated, for multiple immunoassays. For these experiments, two 1 min pulses of 10mM HCl and one 1 min pulse of 5mM NaOH, was found to regenerate the surface reproducibly to within 10 RU of the original baseline value.

4.12.1 Direct immunoassay for CHLDH

Affinity-purified polyclonal anti-CHLDH antibody was immobilised to the sensor chip surface, as outlined in section 2.13.1. The amount of antibody immobilised in terms of response units (RU) was 6879 RU. CHLDH samples, prepared in 0.15M PBS, pH 7.4 (0.009 - 5.000 µg/ml), were injected over the immobilised antibody, as outlined in section 2.13.2.1. A standard curve for the known concentrations of CHLDH is shown in Figure 4.12.3. The graph appears linear for the concentration range 0.312 - 5.000 µg/ml (r=0.997). The Y-error bars are shown on the graph but in most cases are too small to be seen. The CV was less than 4%, over the whole measuring range.

4.12.2 Two-step immunoassay for CHLDH

CHLDH samples prepared as in the previous section, were injected over the immobilised antibody (7531 RU), followed by the same polyclonal anti-CHLDH antibody, to amplify the signal and improve sensitivity, as outlined in section 2.13.2.2. A standard curve for the two-step assay is shown in Figure 4.12.4. Clearly, the standard curve has the same linear range (0.312 - 5.000 µg/ml), as the direct assay (r=0.998), but the response units are approximately 2.8 times higher, due to amplification of the response by the second antibody.

Figure 4.12.1

Sensorgram of typical immobilisation of antibody/antigen to sensor chip surface, as outlined in section 2.13.1. HEPES-buffered saline is allowed to flow over the surface (a-b). The surface is activated with 50mM NHS/ 200mM EDC (b-c) and the antibody/antigen solution in 10mM sodium acetate, pH 5.5, allowed to immobilised to the surface (c-d). Excess reactive groups are inactivated with 1M ethanolamine hydrochloride, pH 8.5 (d-e). A pulse of acid (typically 100mM HCl) is then passed over the chip surface to remove any non-covalently bound material (e-f) and the amount of antibody/antigen immobilised determined in terms of response units, (4317 RU for this sensorgram).

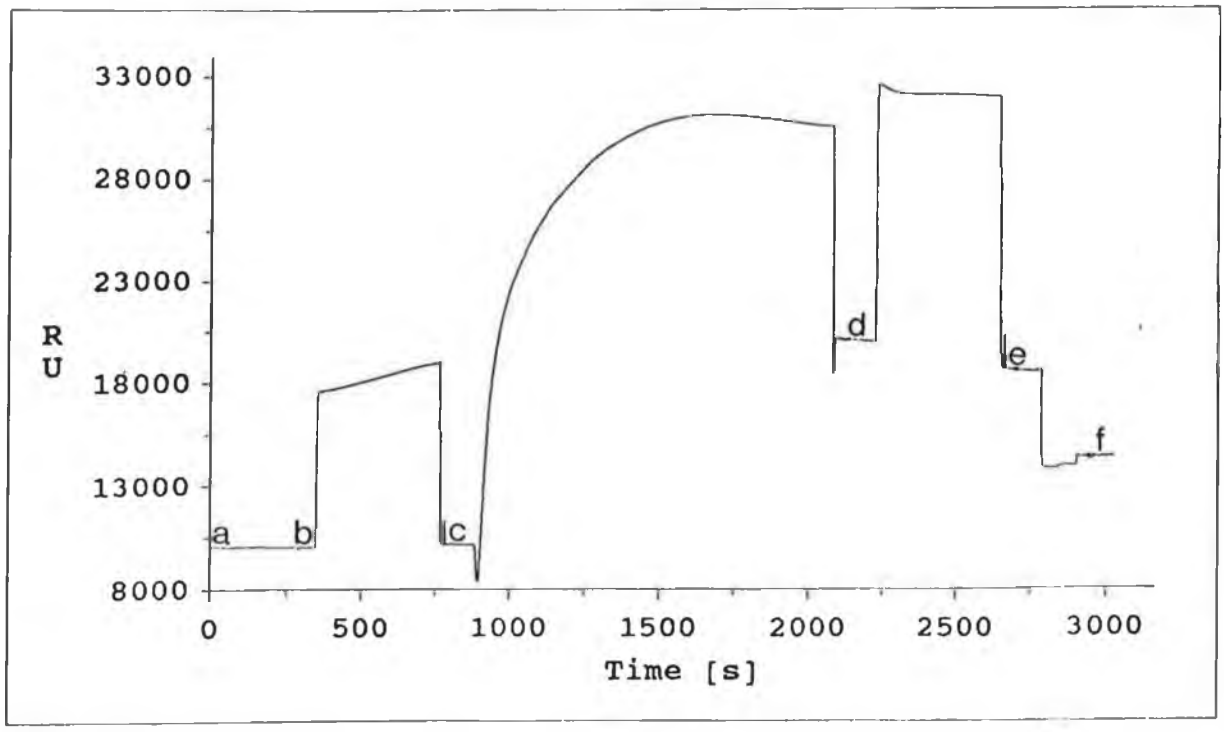


Figure 4.12.2

Sensorgram of typical antibody-antigen binding curve. The baseline is established in HEPES buffered saline (a) and then the antibody (or antigen) passed over the chip surface. The dip from (a-b) is due to the change in refractive index due to the buffer change from HEPES to PBS. The antibody binds to the antigen (b-c) for 20 mins and the total binding determined on returning to HEPES buffer (d). The sensor chip is regenerated using a 1 min pulse of 10mM HCl (d-e), 1 min pulse of 0.1M glycine, pH 2.5 (e-f) and finally a 1 min pulse of 10mM HCl (f-g). A 1 min pulse of HEPES-buffered saline was passed over the chip between regenerations.

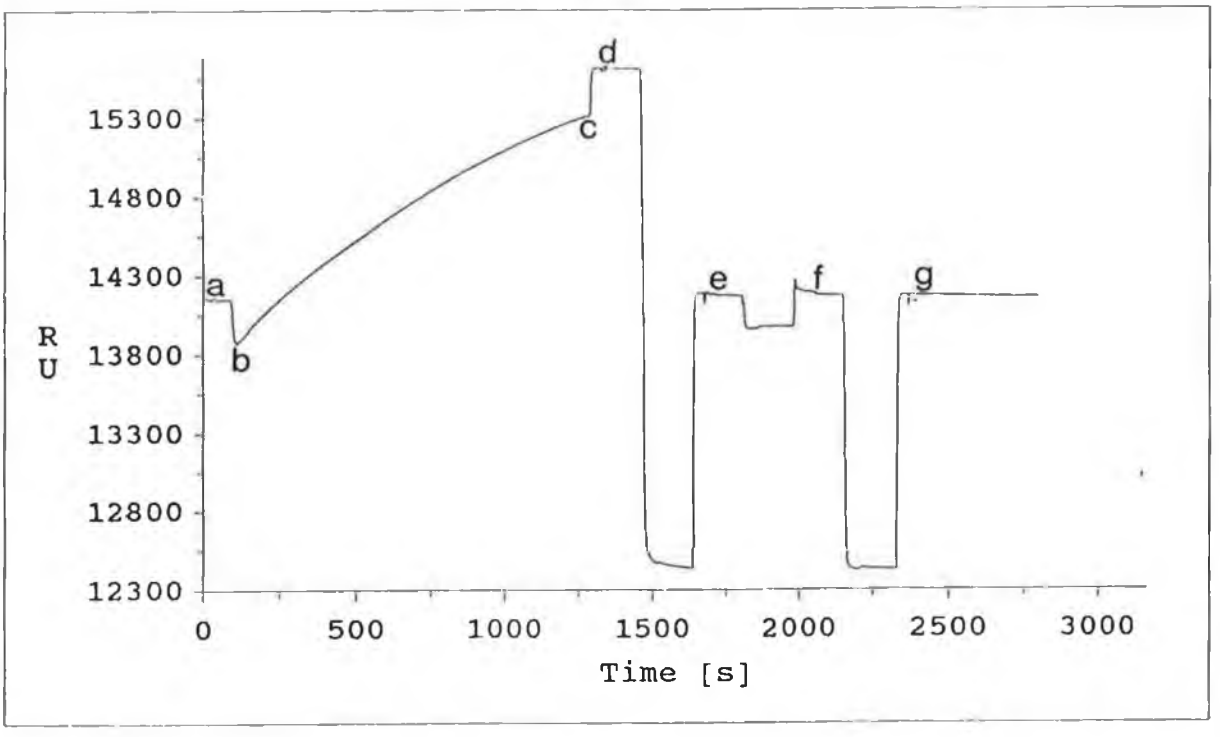


Figure 4.12.3

Standard curve for direct assay for CHLDH using the BIAcore™. The response units for each antigen concentration was determined as the total binding after two minutes (end-point method). The graph is linear for CHLDH concentrations ranging from 0.312 - 5.000 µg/ml (r=0.997). The samples were injected over the immobilised anti-CHLDH antibody, randomly and in triplicate. The coefficient of variation is <4% for all sample concentrations.

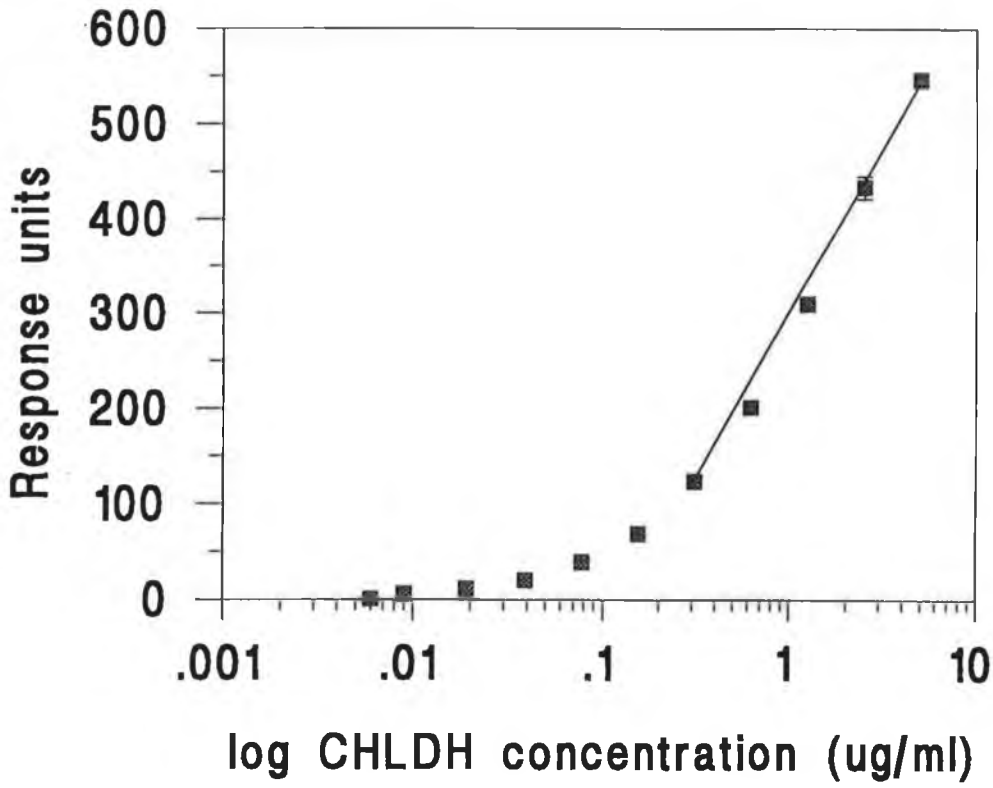
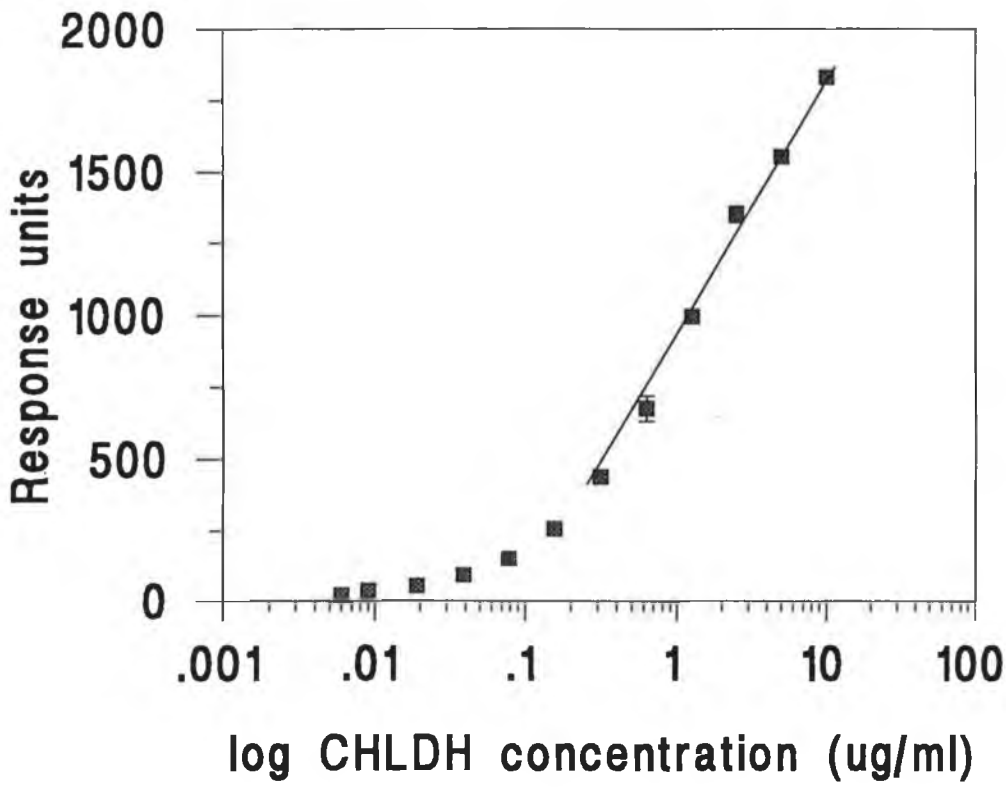


Figure 4.12.4

Standard curve for two-step assay for CHLDH using the BIAcore™. The response signal for each antigen concentration was determined as the total binding after 2 min CHLDH followed by 2 min anti-CHLDH antibody (end point method). The graph is linear for CHLDH concentrations ranging from 0.312 - 10.000 µg/ml (r=0.998). The samples were injected randomly and in triplicate. The coefficient of variation for all the samples was <4%.



4.12.3 One-step assay for CHLDH

CHLDH samples (0.009 - 10.000 µg/ml) were premixed with 20 µg/ml polyclonal anti-CHLDH antibody, and incubated for 1 minute at room temperature, before injecting over the immobilised anti-CHLDH antibody (4253 RU). This process was controlled automatically by the BIAcore™ autosampler and microfluidics system. A standard curve for the one-step assay is shown in Figure 4.12.5. The one-step assay is not as sensitive as the direct and two-step assay. The graph is linear between 1.25 - 10.00 µg/ml (r=0.979), for the range of CHLDH concentrations assayed.

4.13 Effect of increased FITC labelling on antibody affinity, as determined using the BIAcore™ and the evanescent wave immunosensor

Protein A-purified anti-CHLDH antibody, at a concentration of 1.8 mg/ml, was labelled with increasing concentrations of FITC (50, 100, 200, 400, 1600 µg FITC per mg of protein) as outlined in section 2.6.3. The molecular fluorophore to protein ratio was estimated using the formula given in The and Feltkamp (1970), and the results are given in Table 4.13.1. The protein concentrations of all the conjugates was determined using the Bio-Rad protein assay (section 2.7.2), as the FITC appeared to interfere with the BCA protein assay (section 2.7.1). All the FITC-antibody samples were diluted in 0.15M PBS, pH 7.4, to give a final protein concentration of 100 µg/ml. The effect of increased labelling on the affinity of the FITC-anti-CHLDH antibody was assessed using the BIAcore™ and the evanescent wave immunosensor.

4.13.1 Effect of FITC-labelling on anti-CHLDH antibody affinity using the BIAcore™

Antigen (CHLDH) was immobilised to the sensor chip surface, using the immobilisation method outlined in section 2.13.1. The amount of CHLDH immobilised in terms of response units was 4317 RU. FITC-labelled anti-CHLDH antibody (100 µg/ml) was injected over the immobilised antigen as outlined in section 2.13.3, and the resulting sensorgram recorded. The absolute amount of FITC-antibody bound after 20 minutes was plotted against the molecular F/P ratio, and is shown in Figure 4.13.2. With increasing labelling (F/P ratio), the amount of antibody bound to the immobilised antigen decreases.

Figure 4.12.5

Standard curve for one-step assay for CHLDH using the BIAcore™. The response signal was determined after premixing CHLDH with 20 µg/ml anti-CHLDH antibody for 1 min and determining the total binding to the chip after 2 min (end-point method). The graph is linear for CHLDH concentrations ranging from 1.25 - 10.00 µg/ml (r=0.979). The samples were injected over the immobilised antibody randomly and in duplicate. The coefficient of variation for the linear range was <20%.

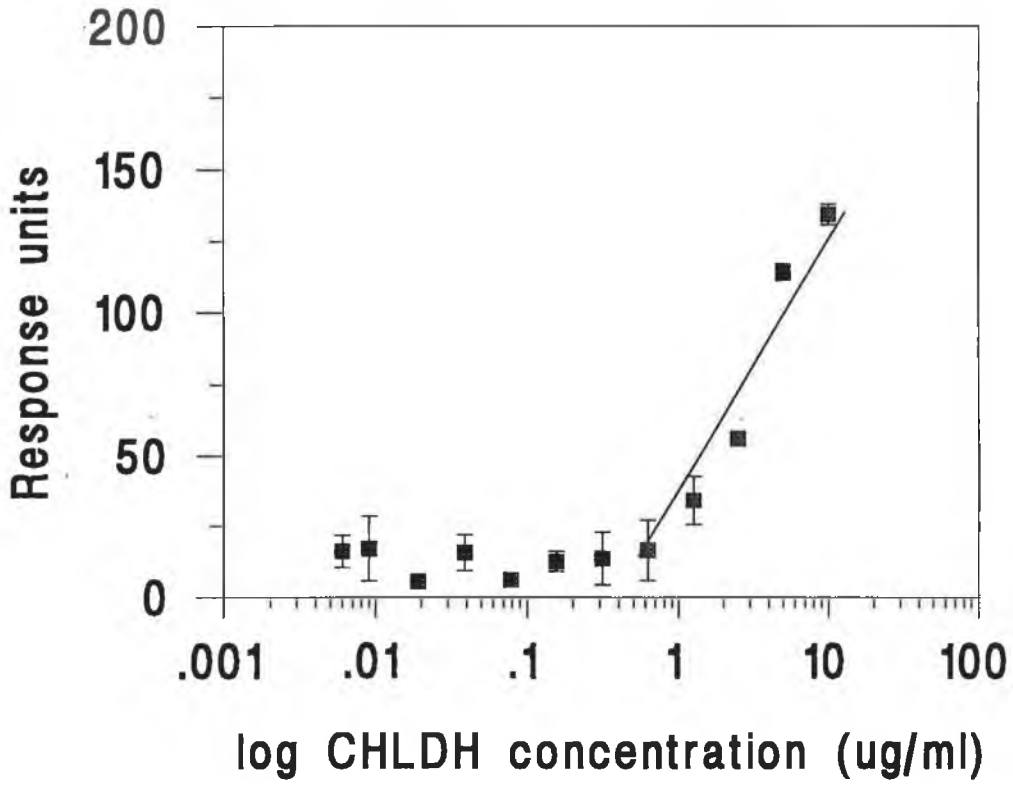


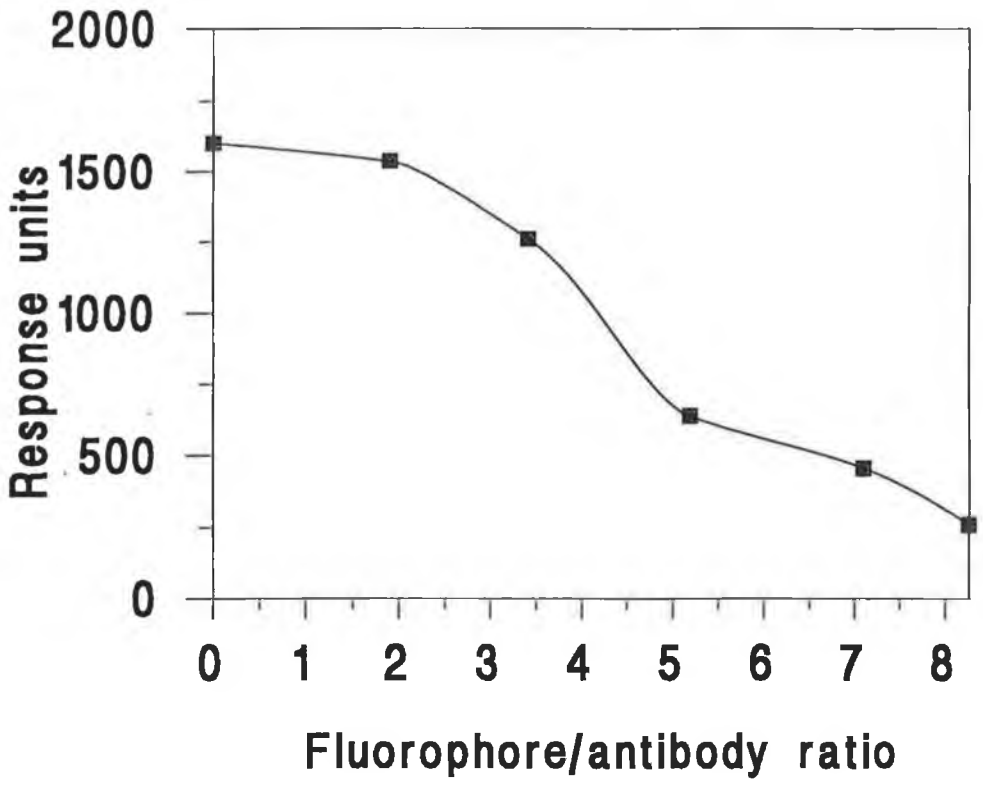
Table 4.13.1

FITC-labelling of protein A-purified anti-CHLDH antibodies using the method as outlined in section 2.6.3. Anti-CHLDH antibody (1.8 mg/ml) was labelled with 50 - 1600 µg FITC per mg protein, and the molecular fluorophore to protein ratio determined using the formula given in The and Feltkamp (1970).

µg FITC per mg protein	*Abs 495 nm / Abs 280 nm*	*Molecular F/P ratio*
50	0.540	1.91
100	0.797	3.42
200	0.992	5.19
400	1.327	7.10
1600	1.436	8.26
control	0.079	0.00

Figure 4.13.2

Effect of increased fluorophore-to-protein ratio on anti-CHLDH antibody binding, as determined using the BIAcore™. The binding was measured in terms of the total response units (i.e. antibody bound) after 20 minutes, to the CHLDH coated sensor chip surface.



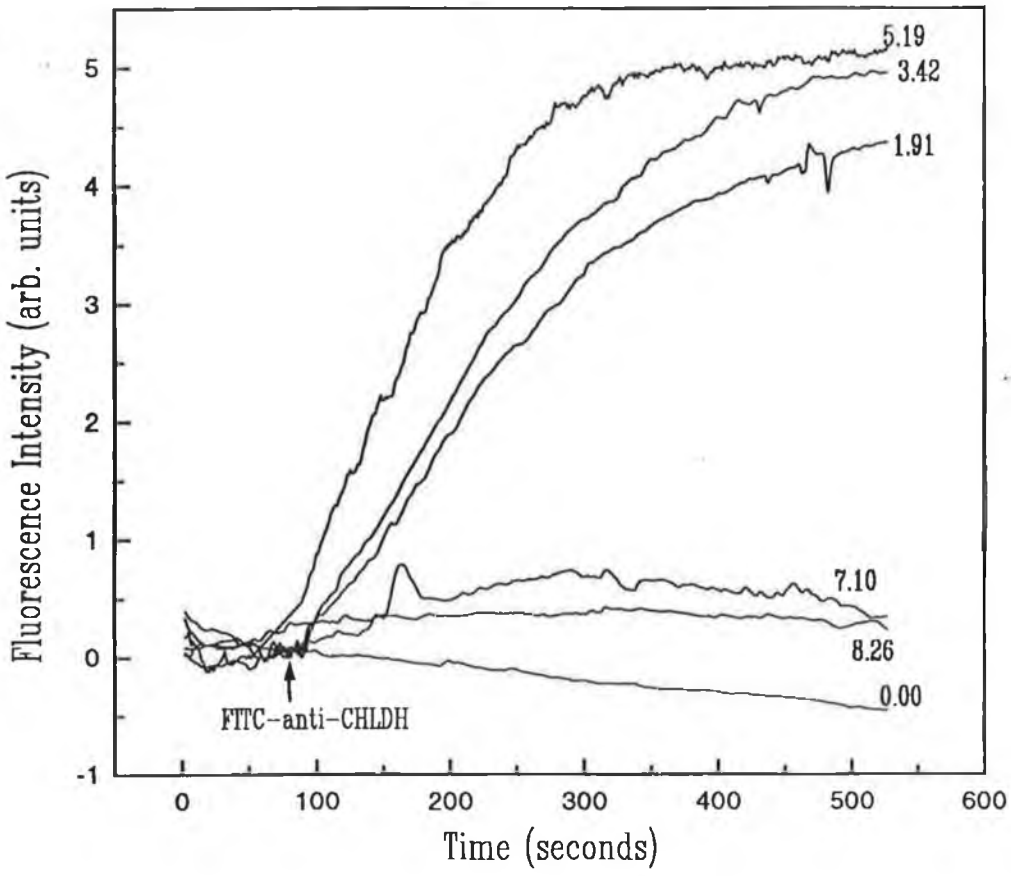
4.13.2 Effect of FITC-labelling on anti-CHLDH antibody affinity using the evanescent wave sensor

Fibres were coated with CHLDH, in the same manner as antibody immobilisation (as outlined in section 2.10.2). FITC-anti-CHLDH antibody (100 µg/ml) was injected into the flow cell and 8.75 minute scans recorded. The scans were overlaid and are shown in Figure 4.13.3. The affinity of the antibody appears to be adversely affected, with high levels of labelling (i.e. greater than 6 FITC molecules per antibody molecule). Very little, if any, binding, to the CHLDH coated fibres, was observed for these antibodies. FITC does not suffer seriously from fluorescence quenching at high fluorophore to protein ratios, as is found with other fluorophores, such as rhodamines (Haugland, 1991). It was concluded, therefore, that the absence of a signal for overconjugated antibodies was due to denaturation of the antibody and not due to quenching at high F/P ratios. The control antibody samples produced no signal, due to the fact that the antibody was not labelled with FITC, and so the binding reaction cannot be transduced and detected.

With lower levels of FITC labelling, i.e. less than 6 FITC molecules per antibody molecule, the antibody retains its ability to bind to its antigen. From the BIAcore™ results, it is clear that the affinity of the antibody decreases with increased F/P ratio. However, the loss in binding affinity is compensated for by the increased amount of FITC available for detection. The signal progressively increases with increased FITC labelling up to 5 fluorophores per antibody molecule.

Figure 4.13.3

Effect of increased FITC labelling on anti-CHLDH antibodies as determined in an evanescent wave fluoroimmunoassay. Anti-CHLDH antibody samples labelled with various levels of FITC (fluorophore-to-protein ratios of 0.00, 1.91, 3.42, 5.19, 7.10, 8.26), as outlined in section 2.6.3, and the effect of increased labelling on the affinity and fluorescence of the conjugates was determined in an evanescent wave fluoroimmunoassay.



4.14 Discussion

Model immunoassay experiments were performed on the surfaces of the fibres, employing HRP-labelled antibodies. These model ELISA's were used to evaluate the optimum coating antibody concentration, the reproducibility of the immobilisation procedure and the activity of the immobilised antibodies. The ELISA assay parameters were chosen where possible, to correspond as closely as possible to the conditions used in the evanescent wave immunosensing experiments. The ELISA employs an end-point method for determination of concentrations whereas the EWS measures concentrations on initial binding slope determinations.

The method of Bhatia *et al.* (1989), was used to covalently immobilise anti-CHLDH antibodies to the silica surface of exposed core optical fibres. Covalent immobilisation was preferred as covalently immobilised antibodies are more stable than adsorbed antibodies (Jönsson *et al.*, 1985). This method was chosen because although it does not allow directional orientation of antibody molecules, crosslinking of antibody molecules to each other is prevented. The heterobifunctional crosslinker has a different functionality at either end of the molecule, one which reacts with the thiol-terminal silane and one which reacts with the protein. The heterobifunctional crosslinker was allowed to react with the silylated fibre first, and then the antibody solution was added. This ensures that the crosslinker does not react with thiol groups on the protein. Bhatia *et al.* (1989), reported that the amount of immobilised antibody on the fibre was 0.96 ng/mm^2, and that the antibody retained its ability to bind 0.37 - 0.55 mole antigen per mole of antibody, as determined by radioisometric immunoassays. This was 3 - 5 times greater than reported by other investigators where the retention of antibody binding activity was less than 10% (Sportsman and Wilson, 1980).

A ratio of 1 fibre per 1 ml of solution was maintained throughout the immobilisation procedure, i.e. for 20 fibres the volume of the silylating, crosslinking and antibody solutions was 20 ml. The vessel geometry was chosen to ensure that the entire exposed core surface of the fibre was immersed in liquid.

The concentration of immobilising antibody to use was assessed by ELISA, so as to be certain that the maximum amount of antibody possible was immobilised to the surface, without wasting the antibody preparation. The actual concentration of antibody immobilised to the surface was not determined as this would involve the use of radioisometric assays, where the specific activity of the radiolabelled conjugates, in

μCi/μg protein, is known (Bluestein *et al.*, 1991). A method which does not require the use of radioactivity was described by Feldman and Uzgiris (1993). They used citric acid to remove bound FITC-labelled antigen from fibre immobilised antibody, and after readjustment of the pH to neutral, determined the equilibrium binding capacity of the fibres.

All EWS experiments were performed under first-order reaction kinetics, and so the fractional occupancy of the immobilised antibody is low during the initial stages of the diffusion-limited process. The antibody surface density does not affect the signal within a certain range of antigen concentrations (Lin *et al.*, 1994), and so the actual concentration of immobilised antibody is not important for these experiments. The maximum amount of protein was immobilised to the fibre, at antibody concentrations of 40 μg/ml or greater. Using concentrations much greater than 40 μg/ml, would result in wastage of the antibody solution. It would also increase the risk of protein-protein interactions and adsorption to the fibre surface rather than covalent interactions (Porstmann and Kiessig, 1992). The use of lower concentrations of antibody and longer incubation times, could attain the same level of immobilisation. However, the GMBS crosslinker is only known to be stable in solution for 1 hour. This was determined by Bhatia *et al.* (1989), using nuclear magnetic resonance.

The 'inter-batch' reproducibility of the immobilisation procedure was also assessed qualitatively by ELISA. The fibre-to-fibre variability as regards the amount of protein immobilised was shown to be less than 4%. Intra-batch variability was not determined, but all the batches were prepared under the exact same experimental conditions and all fibres prepared in a single batch were used for a single experiment.

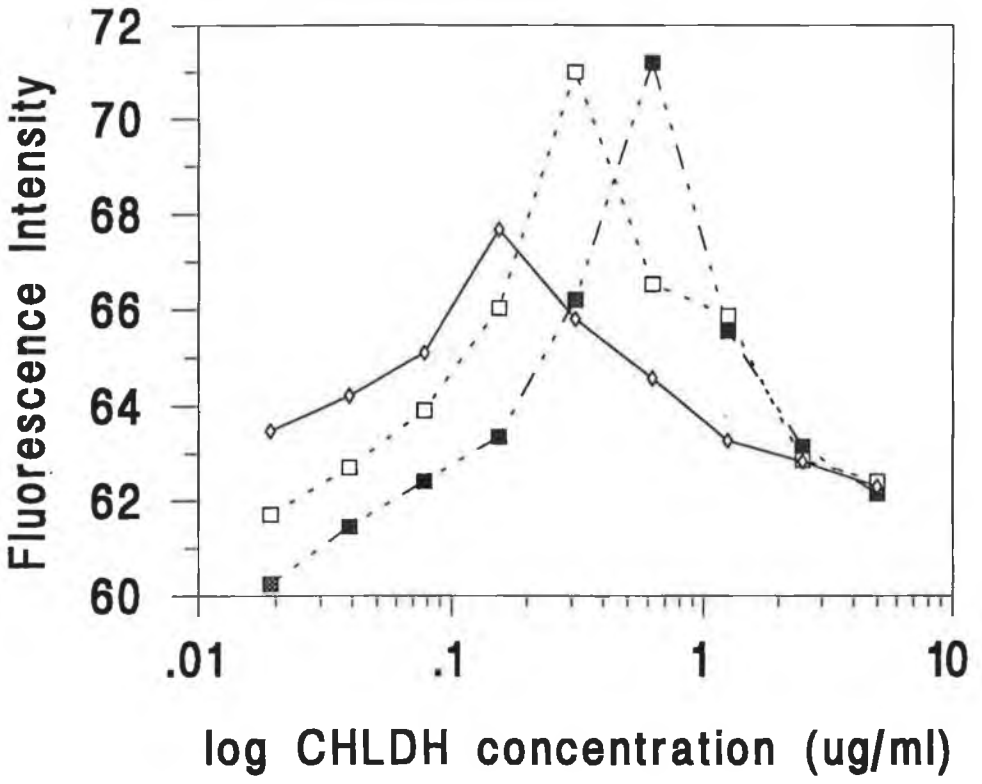
The activity of the immobilised antibody was also assessed by ELISA, under non-equilibrium conditions. The ELISA performed on the fibres, employed the same incubations times and antigen concentrations as the EWS experiments. One-step and two-step ELISA's were performed and compared. For biosensing applications, two-step assay protocols cannot be considered to fulfil the criteria for direct signal readout. Two-step immunoassays require multiple reagent addition steps and user-mediated separation of bound from unbound moieties. Ideally, biosensors should not require any assay manipulation once the sample has been added to the system, resulting in direct sample signal readout (Robinson, 1991). Both immunoassays showed that the antibody retained its biological activity once immobilised to the fibre. The amount of antigen bound

increased exponentially over the range. The linear range for the two-step assay (0.125 - 1.000 µg/ml) was similar to that of the one-step assay (0.05 - 1.00 µg/ml). A decrease in signal was observed (data not shown), for the one-step immunoassay, at higher antigen concentrations. This can be explained by the fact that the free antigen in solution and the antigen in the HRP-antibody/antigen complex compete for binding sites on the immobilised antibody. At high antigen concentrations, the amount of antigen exceeds the amount of labelled antibody and so the amount of free antigen in solution progressively increases over that in the HRP-antibody/antigen complex. The proportion of untagged antigen bound to the fibre increases, and a progressive decrease in the signal results. The linear range of the one-step assay can be increased by either increasing the concentration of immobilised antibody on the fibre, using more highly purified antibodies or increasing the concentration of the labelled antibody. The concentration of immobilised antibody is limited by the surface area of the fibre and the antibodies used here were affinity-purified, and therefore only specific antibodies are present. The only parameter which can be adjusted to improve sensitivity is the concentration of the HRP-labelled antibody. In summary, the concentration of the labelled antibody must be greater than the highest antigen concentration in a one-step assay, to prevent a 'high-dose hook effect'. The effect of increasing the concentration of the labelled antibody is illustrated in Figure 4.14. Walczak *et al.* (1992) stated that at least a five-fold excess of labelled antibody is required to minimize the reaction dependence upon the secondary labelled antibody. The linear range of the assay can be expanded by controlling the labelled antibody concentration.

All the fibre ELISAs were performed in order to characterise the fibre probe surface and to optimise the parameters for the evanescent wave sensing experiments, as much as possible. No direct comparison can be made between the ELISAs and evanescent wave immunosensing since the labelled antibody is different for both, HRP-labelled and FITC-labelled antibody, respectively. The affinity of these antibodies may be different, due to the presence of the label. However, the ELISA experiments are simple to perform and give valuable information about quantity and activity of the immobilised antibody.

Figure 4.14

Illustration of the 'high-dose hook effect'. A one-step fluoroimmunoassay was performed in microtitre plates, using CHLDH at concentrations ranging from 0.019 - 5.000 µg/ml CHLDH and 10 (■), 5 (□) and 2.5 (◊) µg/ml FITC-labelled anti-CHLDH antibody. The fluorescence intensity was measured using the microtitre plate reader facility of the Perkin-Elmer fluorimeter.



Evanescent wave immunosensing experiments were carried out as described in section 2.12.2 with proximal end detection of fluorescence, as outlined in section 2.12.1. Proximal end detection offers many advantages over distal end detection in that the signal-to-background ratio is greatly increased (Yoshida *et al.*, 1988). With distal end face detection of fluorescence, the totally internally reflected incident radiation has to be filtered from the fluorescence which is carried by higher order modes in the fibre. The fluorescent signal generated by the immunoreaction is very low. Typically, only 2% of this is recaptured by the fibre, under optimal conditions (Love *et al.*, 1991). Therefore, a very small signal must be separated from a very large background signal. This is avoided with proximal end face detection.

One of the difficulties with using this system is that the fibre has to be aligned in the optical system, so that the laser light is optimally launched down the fibre, without the aid of an initial fluorescent signal. The excitation and detection of the resulting fluorescence signal was carried out at the proximal end of the fibre and so exact alignment of the proximal end face in the laser beam is important. The launch conditions affect the sensitivity of the system. The detected signal has been shown to be approximately proportional to the area of the illuminated spot on the end face of the fibre (Love and Button, 1988). Glass *et al.* (1987), showed that the signal level from a fibre-optic evanescent fluorosensor is a highly sensitive function of the numerical aperture of excitation and collection, showing an eighth power dependence of sin θ_{max}, the external launch angle. Therefore, if the fibre is not aligned correctly in the optical system, filling all the launch angles, the signal can be adversely affected. Walczak *et al.* (1992), chose a more conservative 25° launch and collection angle in their optical measurements. Although more signal intensity could be obtained at higher angles of up to θ_c (36.8°), the ease of optical alignment and insensitivity to perturbations of the light into solution yielded the more conservative approach. Lin *et al.* (1994) used a moulded polystyrene integrated lens optical fibre, which increases the efficiency of light coupling between the light source, the fibre and the detector. The integrated lens also serves to decrease the positional tolerance within the optical path, thus alleviating the difficulty in alignment of the fibre. The moulded polystyrene also has the added advantage of reducing the effect of V-number mismatch (Anderson *et al.*, 1993), as the mould material also holds the fibre in position.

Alignment reproducibility was found to have a coefficient of variation of approximately

14%, when a one-step assay was repeated 7 times, using a new fibre probe for each assay. This indicates that there is some variability in the alignment procedure. Other variables such as differences in the polishing quality for different fibres, slight variations in the pumping rate etc., may have contributed to the variability but care was taken to reduce these effects.

Antibody-antigen interactions generally have high affinity constants, which confers high sensitivity to immunoassays. The price to be paid, however, is irreversibility. Reversible immunoassays can be developed if the dissociation rate of the antibody-antigen reaction is sufficiently fast, so that the analyte will dissociate in a short enough time to allow rapid re-equilibration to be established in response to a change in concentration. Astles and Miller (1993), reported a reversible immunosensor for phenytoin, based on homogenous fluorescence energy transfer immunoassay. The sensor had a response time of 15 minutes. As an alternative to reversibility, the fibre probes can be regenerated with conditions which will break the antibody-antigen bond, allowing reuse of the fibre probe, for subsequent assay measurements. Physical and chemical variables can be used to manipulate and control antibody antigen reactions, such as surface tension, pH, ionic strength, temperature and the presence of dehydrating agents or chaotropic salts. The conditions for breaking an antibody-antigen bond cannot be predicted, as the nature and strength of the bond is an intrinsic property of a particular antibody-antigen pair, depending on the combination of the amino acid residues found in the binding site. For example, increasing the ionic strength of the buffer works well for dissociating, electrostatic bonds, but where mainly hydrophobic interactions prevail, the opposite is true, and chaotropic salts are required (Jefferies and Deverill, 1992).

The effect of regenerating the immunosensing surface of an aligned fibre was investigated as an alternative to aligning a new fibre for each assay. A single fibre could be used for multiple immunoassays. Relatively good regeneration was obtained when 0.1M glycine/HCl, pH 2.5, was used as the regeneration solution, and the coefficient of variation was <6% for 7 regenerations. This regenerating solution was used to dissociate the antibody from the immunoaffinity support (section 2.4.2.2), during the purification of the antibody. Problems due to air bubbles in the flow system meant it was necessary to realign the fibre at irregular intervals, and so the variability associated with the realignment was introduced. The baseline signal after the regenerating solution was pumped out of the flow cell and refilled with 1% (w/v) BSA, was higher than the initial

baseline signal. It was observed that the baseline increased progressively with further regenerations. This indicated that some of the immunocomplex remained associated with the fibre, with each regeneration. This could be due to the fact that the antibody is polyclonal in nature, and the bonds for the higher affinity antibodies are more difficult to break. More likely however, is the probability that the acidic solution is causing denaturation of the immobilised antibody, resulting in the exposure of hydrophobic regions in the protein. Labelled antibody can non-specifically adsorb to these hydrophobic regions.

Bright *et al.* (1990), regenerated the distal tip of a fibre optic immunosensor with 0.1M phosphoric acid, up to 50 times without significant inactivation of the immunosensing surface. The fibre probe had to be immersed in the acidic solution for 10 minutes for each regeneration. Wijesuriya *et al.* (1994), investigated the regeneration properties of an acidic solution, pH 1.75, containing 50% ethylene glycol, a basic solution, pH 11.0, containing 50% ethylene glycol and a 50% ethanol solution. The mixture of the acid or base with ethylene glycol reduces the attractive forces between the antibody and antigen due to electrostatic interactions and Van der Waals/hydrogen bonding interactions. Hydrophillic proteinaceous antigens were found to dissociate from their antibody, using these buffers, with the exception of very high affinity antibodies. A monoclonal antibody of moderate affinity was found to dissociate cleanly from its hydrophobic antigen with 50% ethanol solution. However, this was attributed to the fact that the antigen was more soluble in the ethanol solution. Different dissociating solutions may have improved the dissociation of the CHLDH from the anti-CHLDH antibody coated fibres. However, due to the fact that realignment was required when air bubbles were introduced into the flow cell and denaturation of the immobilised antibody appeared to be occurring, it was decided to use a new fibre for each assay.

One-step and two-step immunoassays were performed on the fibres and the rate of the initial binding reaction determined using linear regression, as shown in Figure 4.11.5.1. The reaction is pseudo first-order and, therefore, the initial rate of binding is directly proportional to the analyte concentration. The binding kinetics of antigen by immobilised antibody is described by Sadana and Sii (1992).

For antigen (Ag) binding to an immobilised antibody (Ab), the rate of binding is given by;

$$\frac{d\Gamma_1}{dt} = k_1 C_S(\Gamma_0 - \Gamma_1) - k_{-1}\Gamma_1 \qquad (4.6)$$

where

Γ_1 = the surface concentration of antibodies which are bound by antigen at time t

Γ_0 = total concentration of the antibody binding sites on the surface

k_1 = association constant (or forward reaction rate) for Ab-Ag binding

k_{-1} = dissociation constant (or reverse reaction rate) of Ab-Ag binding

C_S = concentration of antigen close to the surface

For initial binding kinetics, $\Gamma_1 << \Gamma_0$, since very few of the binding sites of the immobilised antibody are occupied. Also, $k_1 C_S \Gamma_0 >> k_{-1}\Gamma_1$, since the reverse reaction is very slow for moderate to high affinity antibodies, and dynamic equilibrium is not reached for relatively short times. Therefore;

$$\frac{d\Gamma_1}{dt} = k_1 C_S \Gamma_0 = \frac{d\Gamma_{Ag}}{dt} \qquad (4.7)$$

and so, the initial rate of the reaction is solely dependent on the rate of antigen binding, and the reaction is considered pseudo first-order, as both the capture and labelled antibody are in considerable molar excess.

Diffusion plays an important role in antibody-antigen binding reaction, especially if one of the reactants is immobilised. There is a surface layer (~100 μm thick) which is static and antigen must diffuse across this layer before binding to the antibody. If the reaction rate at the surface is faster than the diffusion rate of the antigen to the surface, the reaction is said to be 'diffusion-limited'. This is assuming there is no flux due to convection, which is true in this case because of the small sample volume (the solution is considered stagnant). The time taken for an immunoreaction to reach equilibrium is dependent on the diffusion coefficient. However, as the rate measurements are to be made during the initial phase of the reaction, there is no need to wait for equilibrium

to occur. Regardless of whether the assay is under reaction or diffusional control, the reaction rate is proportional to the initial bulk concentration of antigen. This means that kinetic measurements are feasible for the measurement of antigen concentrations (Place *et al.*, 1991). In conclusion, the concentration of the labelled and capture antibody must be in excess to ensure that the immunoreaction follows pseudo first-order reaction kinetics, and therefore, is proportional to the antigen concentration. One would only expect deviation from linearity if high analyte levels saturate fibre binding sites quickly bringing about an equilibrium.

The initial binding slope of the curve was determined by linear regression. The first 1 minute of the signal was ignored. This was to remove non-specific fluorescence signals resulting from background fluorescence from the fluorescein content of the bulk solution, which may be coupled back into the fibre by scattering or which diffuses in and out of the evanescent zone by convection (pumping). Fluorescence resulting from non-specific adsorption is also removed. Bluestein *et al.* (1991), reported that 90% of these non-specific effects are removed within the first 10 seconds of signal monitoring. The initial binding rate was then determined over the next one minute of signal monitored, and this was attributed to the immunoreaction. The linear range of the two-step assay was wider than that of the one-step assay due to the hook effect as described earlier. Increasing the labelled antibody concentration should increase the linear range of the one-step assay.

The one-step assay gave much lower overall binding rates than the two-step assay. Shriver-Lake *et al.* (1993), compared the detection sensitivity of a one and two-step assay. The one-step assay employed different monoclonal antibodies as the capture and labelled antibodies, which bound to non-interfering epitopes of a small molecule, pseudexin (m.w. 16,500). For the one-step assay, the antigen and labelled antibody are allowed to bind in solution, forming a FITC-antibody/antigen complex, which on pumping into the flow cell binds with the immobilised capture antibody. By using non-interfering monoclonal antibodies, the binding of the antigen to the fibre immobilised antibody is not blocked. The two-step assay was performed using the same batch of polyclonal antibodies against a large molecule, botulinum toxin (m.w. 150,000), for the preparation of the capture and labelled antibody, the capture antibody being affinity-purified. The overall signal for the two-step assay was lower than the one-step assay. This reason for this was attributed to the fact that the blocking of some of the epitopes

by the capture antibody reduces the number of epitopes for the second antibody, and hence, the rate of binding.

CHLDH is a large molecule (m.w. 140,000), and so the polyclonal antibodies raised against it bind to many epitopes on its surface. This means that for the two-step assay, there is a sufficient number of epitopes remaining for the labelled antibody to bind, after the antigen has bound to the capture antibody. For reasons cited earlier, there is a reduced number of epitopes available for the capture antibody to bind, in a one-step assay, thereby reducing the rate of binding. There is also the factor of steric hindrance, the labelled antibody preventing easy access of the capture antibody. The diffusion coefficient for the larger complex for the one-step assay is also decreased, thus affecting the rate of binding.

Both the one-step and the two-step immunoassay for CHLDH, gave equivalent sensitivity (0.03 µg/ml), although the magnitude of the response was very different. For CHLDH, concentrations lower than 0.03 µg/ml, no signal over background was detected. Shriver-Lake *et al.* (1993), achieved much lower detection limits (1 ng/ml), using tapered optical fibres, which improves fluorescence detection (Anderson *et al.*, 1993).

A competitive immunoassay was performed using FITC-labelled CHDLH. A fixed concentration of FITC-CHLDH (20 µg/ml) and varying concentrations of CHLDH, competed for binding to the immobilised anti-CHLDH antibody, resulting in an inverse relationship between signal slope and antigen concentration. The working range of the assay, that is the region of the steepest part of the concentration-response curve, was 1.0 - 10.0 µg/ml, using 20 µg/ml, as the labelled antigen concentration. The working range could be adjusted by varying the concentration of the immobilised antibody (which is fixed due to the limited fibre surface area), or the concentration of the labelled antigen (Oroszlan *et al.*, 1993). A lower detection limited can be achieved by lowering the concentration of the FITC-labelled CHLDH. At lower antigen concentrations i.e. <1.0 µg/ml, the labelled antigen concentration is too high to allow the difference in the fluorescence signal to be measured. Concentrations greater than 10 µg/ml were not measured.

Finally, the effect of solution viscosity on the rate of antibody-antigen binding was tested. It was expected that for higher viscosity solutions, the rate of diffusion would be lowered and, hence, the rate of binding would be decreased. The opposite result was obtained, in that the rate of signal accumulation increased with increased solution

viscosity. A possible explanation for this is that the increased signal is not due to increased rate of accumulation but rather more efficient coupling of the fluorescence back into the fibre. Love *et al.* (1991) showed, using sucrose solutions, that the fluorescence signal was proportional to $N.A._{material}^{-4}$. In other words, the $N.A._{material}$ decreases with increased viscosity, and as the $N.A._{material}$ decreases the signal increases (Equation 4.4).

The BIAcore™ allows direct monitoring of biomolecular interactions without the requirement for labelling one of the biocomponents involved (section 1.4.3.2.2). Furthermore, the dextran strands of the immobilisation matrix, are mobile in three directions, and approximate more closely to a volume rather than an area. Immobilised biomolecules are thus, in effect, 'still in solution'. These properties of the BIAcore™ allows the determination of the kinetic properties of antibodies (Johne *et al.*, 1993), under real conditions, as the immobilisation of antibodies to conventional solid surfaces can alter the association and dissociation properties of the antibody molecules. The determination of the concentration of one of the binding partners can also be carried out. An assay procedure for the determination of the concentration of β_2-microglobulin (Application note 201, Pharmacia Biosensor AB), using a monoclonal antibody as the immobilised molecule and polyclonal second antibody to amplify the signal was reported. Real-time biospecific interaction analysis using a direct (no amplification second antibody) or multi-step binding technique is suitable for concentration determination of proteins with large molecular weights (above about 10,000 Da). For smaller molecules, such as theophylline, a competitive binding assay can be used, as the total mass of analyte which can be bound to the sensor chip surface is too small for confident direct measurement, and sandwich immunoassays cannot be applied when the analyte lacks multiple antigenic sites or epitopes (Application note 202, Pharmacia Biosensor AB).

A direct, two-step and one-step immunoassay for CHLDH was set up on the BIAcore™, in order to compare the immunoassays, with those set up on the evanescent wave sensor (EWS). A direct assay can be set up because the molecular weight of CHLDH is large, 140,000 Da, and on binding to the immobilised antibody, the mass change is sufficient to be transduced directly without signal amplification. With signal amplification (two-step assay), the sensitivity of the assay is increased almost two-fold. A higher level of

sensitivity should have been obtained, had the capture and amplifying antibody not been from the same polyclonal source. The use of the same polyclonal antibody results in some epitope blocking, thereby reducing the number of sites available for the second antibody to bind. This was elaborated on earlier (section 4.12), when discussing one-step and two-step evanescent wave immunoassays.

The detection range of the one-step assay for CHLDH is much narrower when determined on the BIAcore™ compared to the EWS. No binding was observed for concentrations less than 1.0 µg/ml. The reason for this may be that when the CHLDH and anti-CHLDH antibody was premixed by the BIAcore™ autosampler system, the sample was allowed to incubate for 1-2 min before passing over the immobilised antibody, whereas the premixed sample was injected over the fibre probe immediately using the EWS. A higher percentage of the epitopes may have been blocked, due to this incubation. It is also possible that the antibody-antigen complexes may have precipitated out of solution, at these lower concentrations of antigen. A lower quantity of anti-CHLDH was immobilised to the chip surface for the one-step assay (4253 RU) than the two-step assay (7531 RU), which may also have contributed to the lower sensitivity. For higher concentrations of CHLDH, no high dose hook effect is seen as with the EWS. This is because the antigen is detected directly on the basis of its mass and not indirectly via a labelled antibody. Even when the second antibody is no longer in excess, the uncomplexed antigen can still bind to the immobilised antibody and cause a mass change.

Comparing of the one-step and two-step immunoassays as performed on the BIAcore™ and EWS, one can conclude that the one-step assay, which fulfils the criteria for biosensing, can be performed with polyclonal antibodies for large antigens, but with a large loss in detection sensitivity. Using polyclonal antibodies raised in different species may be the answer, as the range of epitopes for the different polyclonals may be different. Production of monoclonal antibodies may be time consuming, expensive and difficult. This was shown using anti-mouse IgG antibodies in a fluorescence-linked immunosorbent plate assay (results not shown). Rabbit anti-mouse IgG (Sigma) and sheep anti-mouse IgG (Dako) were used to coat a microtitre plate and a one-step assay using rabbit FITC-anti-mouse IgG as the second antibody was set up. The signals obtained for the one-step assay using the sheep and rabbit raised antibody as the capture and second antibody respectively, was on average 2-fold higher than the assay which

employed rabbit-raised polyclonal antibodies for both the capture and second antibody. One useful application of the BIAcore™, in the development of biosensors, is the selection of low affinity monoclonal antibody clones, for the development of reversible biosensors.

The BIAcore™ and the EWS were used to determine the effect of increased FITC-labelling on the affinity of protein A-purified anti-CHLDH antibody. The optimal conditions for the conjugation of FITC to antibodies have been described in Goding (1983), to obtain the optimal labelling ratio of 1-4 FITC molecules per antibody molecule (The and Feltkamp, 1970a). The lowest degree of conjugation which gives the maximum fluorescence is preferred because it will cause the least change in the physical and biological properties of the native protein. With high FITC to protein ratios, the isoelectric point of the protein is decreased, which for immunohistochemical staining can cause non-specific binding, especially to acidophillic tissue components (The and Feltkamp, 1970b). However, the optimal degree of conjugation immunohistologically may not be the optimal degree in the physicochemical sense, or for use in fluorescence-linked immunosorbent assays.

Fluorescein isothiocyanate (FITC), probably conjugates to antibodies via the free amino groups of the lysine residues. It is not possible to predict, however, how many lysine residues are in the binding site of an antibody. At high levels of conjugation, it is more probable that an FITC molecule will react with a lysine residue near or in the binding site, causing conformational changes and, hence, affecting the ability of the antibody to bind to its antigen. The effect of FITC conjugation on antibody activity is reviewed in Ward and Fothergill (1976). Above a certain F/P ratio, it has been shown that the fluorescence of the conjugates does not increase. This was explained by the fact that the first fluorophore to attach to the protein will react with the most readily available site, which is most likely to produce least distortion to the protein molecule and the fluorophore structure. Additional fluorophore molecules are more likely to conjugate with less readily available lysine groups, and be more intimate with the protein, and thus be more liable to quenching.

The effect of increasing the FITC/protein ratio, on the affinity of anti-CHLDH antibody, was determined, using the BIAcore™ and the EWS. Abraham *et al.* (1991), studied the influence of periodate oxidation on monoclonal antibody avidity and immunoreactivity. This involved the use of Scatchard analysis and manipulation of inhibition binding data.

Real-time analysis allows much quicker assessment of such parameters. The BIAcore™ monitors the binding of antibody to antigen in real-time. The results clearly show that as the F/P ratio increases, the affinity of the anti-CHLDH antibody for CHLDH decreases. The total anti-CHLDH antibody bound to the CHLDH-coated sensor chip surface after a 20 min incubation, decreased with increasing F/P ratios. This is probably due to the progressive denaturation of the antibody, as the number of FITC molecules bound to it increases, resulting in a reduction in its binding ability. At low levels of conjugation, 2-4 FITC/antibody, the antibody affinity is only reduced by 20%, compared to the control antibody. For higher levels of labelling, 5-8 FITC/antibody, the reduction in affinity is greater than 60%.

The BIAcore™ detects antibody-antigen binding on the basis of mass accumulation, and therefore, does not require a label species for transduction of the antibody-antigen interaction. Therefore, no conclusion could be drawn from the BIAcore™ results about how the fluorescence intensity of the conjugates, could compensate for the loss of antibody affinity at high labelling ratios, in for example, fluoroimmunoassay situations. The EWS was used to determine the affinity of the FITC-labelled anti-CHLDH antibody conjugates by measuring the increase in the fluorescence signal as the immunoreaction occurred. The results show that although the affinity of the antibody labelled with 5.19 FITC molecules per antibody molecule, is only 40% of the control (unlabelled) antibody (as determined by the experiments performed on the BIAcore™), the high level of fluorophore labelling compensates for this, and the steepest binding curve was obtained for this F/P ratio using the EWS. The lack of signal by the even higher labelled antibodies must be attributed to a combination of fluorescence quenching and reduction in antibody affinity. The optimal ratio for fluoroimmunoassay experiments was taken to be 2-5 fluorophores per antibody protein molecule.

CHAPTER 5

DEVELOPMENT OF AN ELISA FOR THE H-SUBUNIT OF HUMAN LDH AND ITS USE IN THE DIAGNOSIS OF AMI

5.1 Measurement of Lactate Dehydrogenase (LDH)

Lactate dehydrogenase (EC 1.1.1.27) catalyses the reaction;

$$\text{Lactate} + NAD^+ \rightleftharpoons \text{pyruvate} + NADH + H^+$$

The activity of LDH can be measured spectrophotometrically. The equilibrium of the above reaction lies far on the side of lactate and NAD^+ (Gay *et al.*, 1968). The cofactor in the reaction, NADH, absorbs strongly at 340nm, and therefore the activity of LDH can be measured by the decrease in absorbance at 340 nm, as NADH is converted to NAD^+. An important feature of this assay is that the optimal pyruvate concentration varies with pH, temperature, isoenzyme and species. Pyruvate is a potent inhibitor of the reaction at high concentrations.

LDH can also be measured by the forward reaction, under suitable reaction conditions. The NADH formed in the reaction can be measured directly by an increase in the absorbance at 340 nm (Amador *et al.*, 1963; Gay *et al.*, 1968), or after coupling to the reduction of a tetrazolium salt (INT), with phenazine methosulphate (PMS) as an intermediate electron carrier, resulting in a strongly coloured product, formazan (Babson and Philips, 1965; Nachlas *et al.*, 1960). Formazan absorbs strongly at 500 nm.

$$\text{Lactate} + NAD^+ \rightleftharpoons \text{Pyruvate} + NADH + H^+$$

$$NADH + PMS + H^+ \rightarrow NAD^+ + \text{Reduced PMS}$$

$$\text{Reduced PMS} + \text{Tetrazolium salt} \rightarrow \text{Formazan} + PMS$$

Alternatively, LDH activity can be measured fluorimetrically. NADH, a product of the forward reaction is strongly fluorescent at 456 nm when excited at 340 nm. Practically however, interference occurs from naturally occurring constituents of serum which will either quench the fluorescence of NADH or fluoresce in a similar region to NADH, causing interference. The inner filter effect of serum also restricts the application of direct enzyme analysis in serum by fluorescent methods. Passen *et al.* (1966), obviated the problems of light scattering, inner filter effects, quenching and non-specific fluorescence by dialysing out the NADH and then measuring the fluorescence.

5.2 Isoenzymes of LDH

Lactate dehydrogenase (LDH), is present in the human body, in five different tetrameric isoenzyme forms. These isoenzymes are composed of different combinations of two different LDH subunit types, H and M (Plagemann *et al.*, 1960). The two forms have different catalytic, physical and immunological characteristics, and are under separate genetic control. The five tetrameric isoenzymes are H_4, H_3M, H_2M_2, HM_3 and M_4 (alternatively LDH_1 - LDH_5, respectively). The isoenzymes are distributed throughout the body, different tissues having a specific isoenzyme pattern of LDH. All five isoenzymes are represented in normal human serum. $LDH_{1,2}$ predominate in tissues such as the heart, kidney, brain, pancreas and erythrocytes while $LDH_{4,5}$ predominate in the tissues of the liver, skeletal muscle and granulocytes.

The isoenzymes of LDH are distinguishable by their chemical composition, kinetic and immunological properties (Cahn *et al.*, 1962). LDH_1 has a low Km for pyruvate and is strongly inhibited by it, whereas LDH_5 has a higher Km for pyruvate and is not inhibited by it. The isoenzymes can be separated from one another electrophoretically, LDH_1 being the most fast-moving, migrating towards the anode while LDH_5, is the slowest moving, migrating toward the cathode. Antiserum against the M_4 isoenzyme of a species will not cross react with its own H_4 isoenzyme. H_4 is a poorer immunogen and may cross react with M_4 from other species.

5.3 Myocardial infarction and LDH

When a region of the heart muscle becomes starved of blood, due to a blockage in an artery leading to the heart, the cells of this region die and acute myocardial infarction (AMI) is said to have taken place (Higgins *et al.*, 1994). It is generally considered that such tissue injury can cause leakage of intracellular enzymes into the circulatory system. A consequence of cell death due to myocardial infarction is that the myocardial cell membranes soon rupture leading to the release of a variety of intracellular enzymes such as creatine kinase (CK), lactate dehydrogenase (LDH) and glutamic oxaloacetic transaminase (GOT), into the circulation. This results in a superimposition of the tissue LDH isoenzyme pattern upon the normal serum isoenzyme pattern. AMI can be treated successfully if it is diagnosed rapidly. Analysis of the LDH isoenzyme pattern in the serum is one method of diagnosis.

5.4 Diagnosis of AMI by isoenzyme analysis

LDH isoenzyme analysis is used extensively for the diagnosis of AMI, liver disease and myopathies since the total LDH concentration in serum is significantly increased for these diseases and the isoenzyme profile of the tissues involved, represent the electrophoretic extremes (i.e. an increase in LDH_1 and LDH_2 or an increase in LDH_5 and LDH_4), and are easily recognised. Other isoenzyme profiles are representative of other diseased states such as cancer. Jacobs *et al.* (1977), reported the clinical significance of the 'isomorphic' pattern of LDH. The isomorphic pattern is defined as a significant increase in the total LDH concentration, with normal or low percentages of individual isoenzyme fractions, and with the LDH_1/LDH_2 ratio less than unity. The isomorphic pattern can result from a wide range of different diseases including cardio-respiratory diseases, malignancy, fractures and infections.

The heart muscle cells predominantly contain the LDH_1 isoenzyme whereas serum predominantly contains LDH_2 (Nisselbaum and Bodansky, 1961). After an episode of AMI, serum LDH_1 increases at 8-12 hours, reaches a maximum at 48-72 hours and returns to normal at 8-12 days later. Acute MI is therefore accompanied by elevated total LDH, reversed LDH_1/LDH_2 ratio and an elevation of LDH_1.

Diagnosis of AMI on the basis of the LDH_1/LDH_2 ratio flip, as determined by electrophoretic methods is commonly used. The isoenzymes are separated by electrophoresis on an agar gel and the zones of activity visualised by performing a chemical reaction where the substrate yields an insoluble chemical product in the presence of the enzyme. The relative activity of each isoenzyme is measured by scanning densitometry (Rosalki, 1974). There was much debate in the late 1970's, however, as to whether the determination of the concentration of LDH_1 alone was better for more rapid AMI diagnosis. Usategui-Gomez *et al.* (1979), reported that for 25% of patients diagnosed with MI, the concentration of LDH_1 was of diagnostic value one day earlier than the LDH_1/LDH_2 isoenzyme ratio. The determination of LDH_1 concentration was carried out using immunochemical techniques. A polyclonal antibody against the M-subunit of LDH and a polymer-conjugated second antibody promoted precipitation of all the isoenzymes containing the M-subunit (M_4, HM_3, H_2M_2, and H_3M), which were removed by centrifugation, allowing the activity of the LDH_1 (H_4), to be determined by spectrophotometric methods. Gerhardt *et al.* (1983), also found that the quantitation of serum LDH_1, was diagnostically more reliable for MI than LDH_1/LDH_2 ratio. Serum

LDH_1 elevation occurs before the ratio of LDH_1/LDH_2 is greater than unity, thus allowing more rapid diagnosis. Weidner (1977), felt that both measurements were important.

A similar method for the determination of LDH_5 with monoclonal antibodies immobilised on latex beads was reported by Vaidya *et al.* (1986).

Results

5.5 Miniaturised colourimetric assay for total LDH

Standard human LDH samples (0.0 - 2.9 µg/ml) were prepared in 1% (w/v) BSA, and the miniaturised assay for total LDH performed as outlined in section 2.14. These LDH sample concentrations span across the normal and abnormal range for total serum LDH values.

The results of the miniaturised assay are shown in Figure 5.5.1. The error bars on the data are present but are too small to be seen. Excellent linearity was obtained over the whole range (r=0.999).

Clinical patient samples (obtained from St. James Hospital) were assayed for total LDH, using the miniaturised colourimetric assay outlined in section 2.14. The activity of the LDH (as determined by automated SMAC analyser in St. James Hospital, Biochemistry Dept.) was plotted against the absorbance values obtained for the miniaturised assay. From Figure 5.5.2, it can be seen that the miniaturised colourimetric assay based on the forward reaction gives good correlation (r=0.983) with the automated analyser, which determines LDH activity based on the reverse reaction.

5.6.1 Production and purification of anti-H_4LDH antiserum

Antisera to human LDH (H_4/LDH-1 isoenzyme), was obtained by immunisation of a New Zealand White rabbit (section 2.2.2). The IgG fraction of the antiserum was purified by ammonium sulphate salt precipitation followed by protein A (SpA) affinity chromatography, as outlined in sections 2.4.1 and 2.4.3. Protein A (isolated from *S. aureus*) binds to the Fc portion of class G immunoglobulins (Surolia *et al.*, 1982), and so isolates specific and non-specific IgG. Results of antiserum titre studies are shown in Figure 5.6.1. The titre was shown to be 1:2000.

Figure 5.5.1

Miniaturised colourimetric assay for LDH. The assay was performed in 96-well microtitre plates, as outlined in section 2.14. The assay is linear for LDH concentrations in the range 0.0 - 2.9 µg/ml (r=0.999).

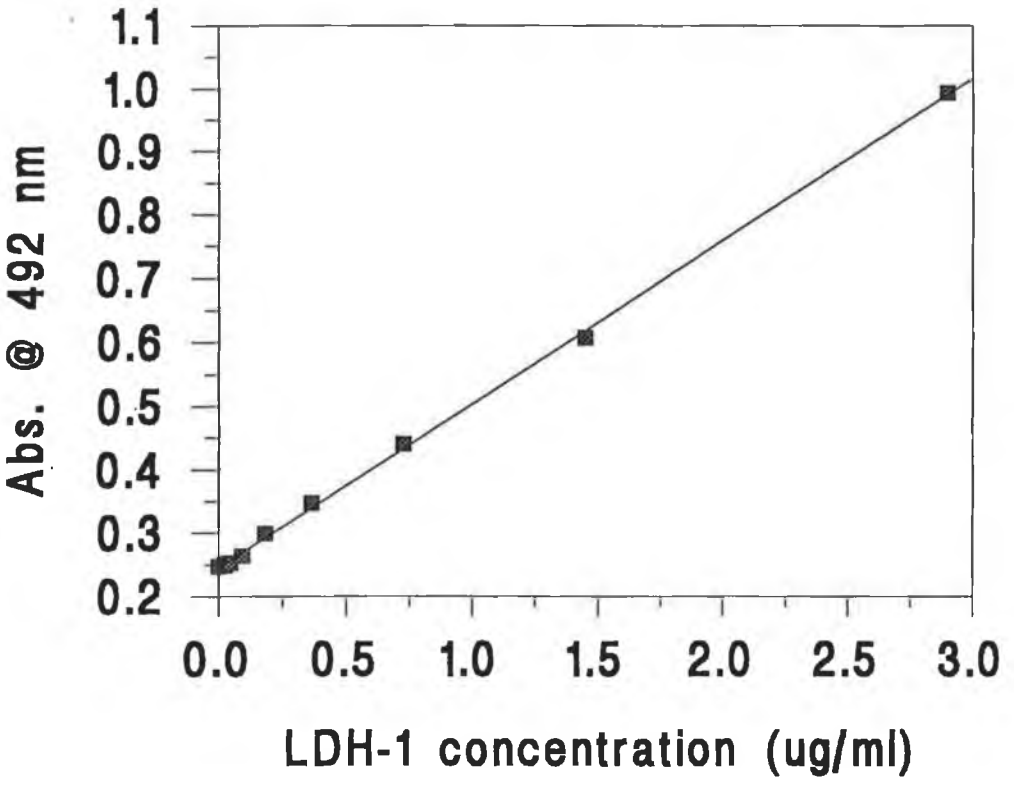


Figure 5.5.2

Correlation between the miniaturised colourimetric assay for LDH (section 2.14), and the LDH activity in patient serum samples, as determined by the automated SMAC analyser (performed in St. James' Hospital). The activity of the LDH is plotted against the absorbance at 492 nm obtained in the minaturised assay. The correlation between the two assays is excellent (r=0.983).

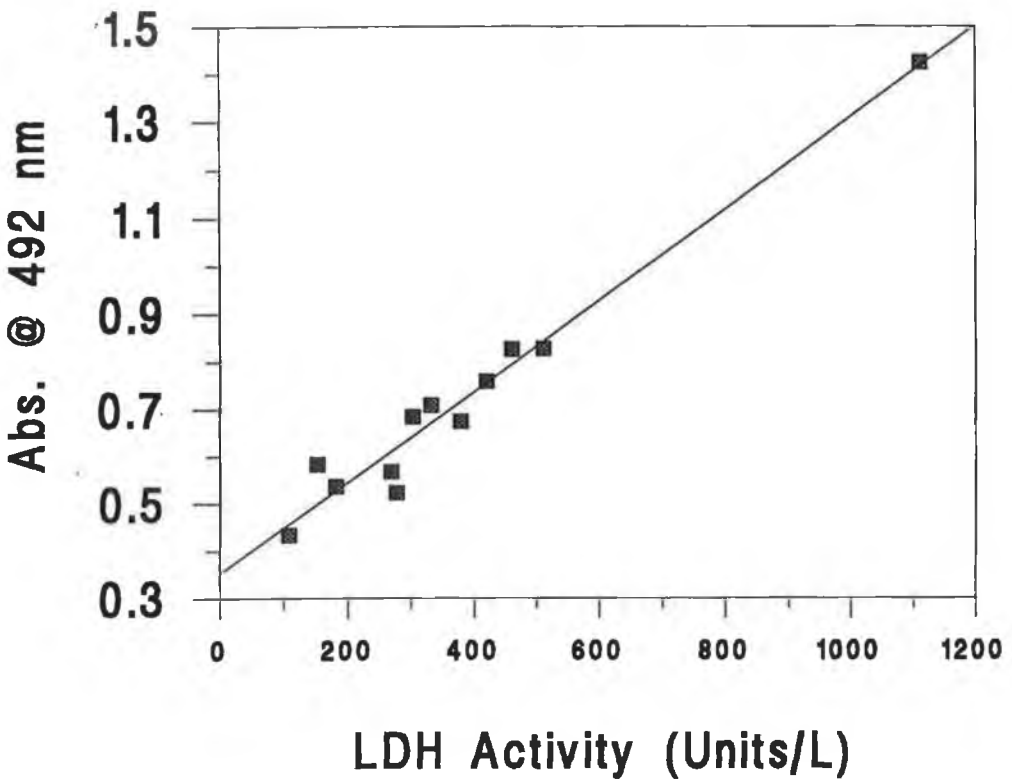
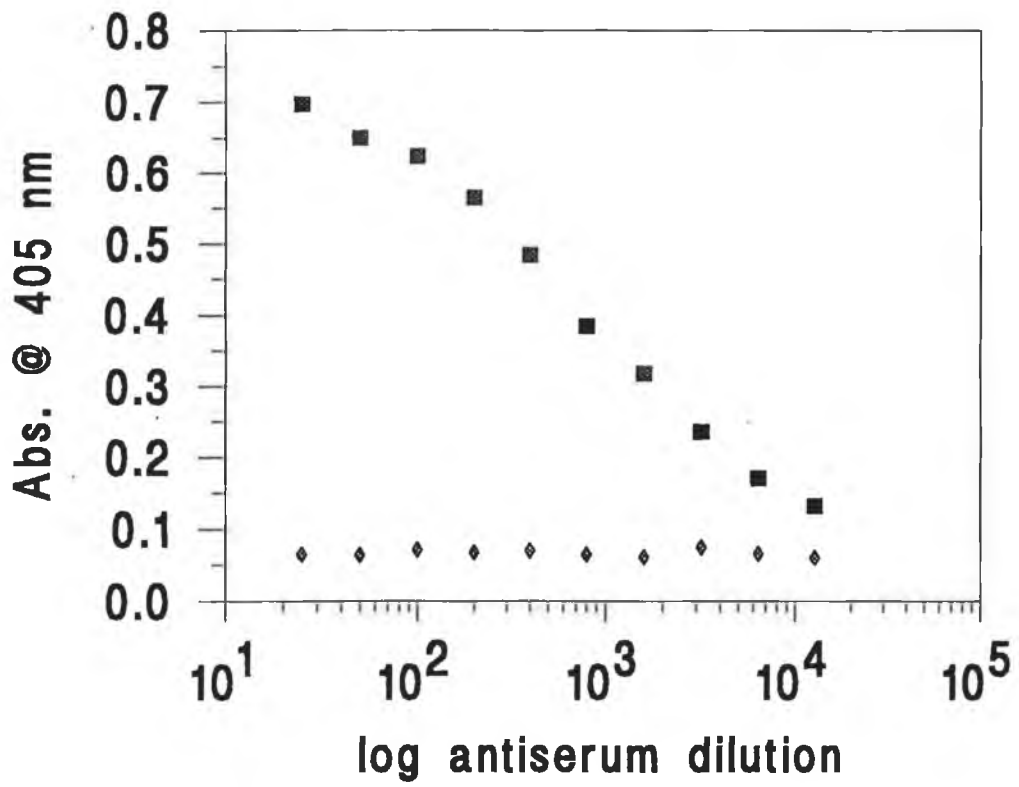


Figure 5.6.1

Titre of purified anti-H_4LDH antibodies. Serial dilutions (1:25 - 1:12,800) of normal rabbit serum (♦), and protein A affinity-purified anti-H_4LDH antibodies (■) were prepared in 0.15M PBS, pH 7.4, and the titre determined as outlined in section 2.5.1. The purified antibody has a titre of 1:2000.



5.6.2 Characterisation of the purified anti-H_4LDH antibodies by SDS-PAGE

The purity of the SpA purified anti-H_4LDH was assessed by SDS-PAGE electrophoresis, as outlined in section 2.8. The SpA purified anti-H_4LDH antibody, SpA purified anti-CHLDH and a commercially available rabbit anti-mouse IgG (IgG fraction) were electrophoresed under non-reducing conditions, in a 5-20% gradient gel and stained with Coomassie Brilliant Blue. The molecular weight markers are shown in Lane 1 (Figure 5.6.2), with rabbit anti-mouse IgG (Lane 2), rabbit anti-CHLDH antibody (Lane 3) and rabbit anti-H_4LDH (Lane 4). The heavy band (the gel was slightly overloaded) for all three samples corresponding to a molecular weight of 160,000-180,000 Da, found between molecular weight markers 112,000 Da and 195,000 Da, represents the IgG fraction. Two fainter bands are also visible for molecular weights, slightly greater than 63,000 Da (possibly BSA, m.w. ~66,000), and slightly smaller than 52,000. These bands appear for the commercial and 'in-house' produced antibodies. This was not expected as Protein A chromatography should bind IgG alone. However, the amount of contaminants compared to IgG was small, and the 'in-house produced' antibodies compared favourably with the commercial preparation.

5.6.3.1 Conjugation of anti-H_4LDH to horseradish peroxidase (HRP) enzyme

Protein A purified anti-H_4LDH was conjugated with the enzyme HRP, as outlined in section 2.5.2. The HRP-anti-H_4LDH conjugate and unconjugated antibody, was purified from the unconjugated enzyme by salt precipitation as outlined in section 2.4.1, and diluted (1:1) with glycerol. The glycerol stabilises the conjugate, allowing it to be stored at -20°C, without freezing.

5.6.3.2 Determination of the working dilution of HRP-anti-H_4LDH antibody conjugate

The working dilution of HRP-anti-H_4LDH was determined as outlined in section 2.5.3. Serial dilutions (1:25 - 1:6400) were prepared in 3% (w/v) BSA in 0.15M PBS-Tween, pH 7.4. The working dilution of the conjugate was determined to be 1:100 (Figure 5.6.3).

Figure 5.6.2

Analysis of the purity of the protein A affinity purified antibody, by SDS-PAGE (5-20% gradient gel), under non-reducing conditions, and stained with Coomassie Brilliant Blue (section 2.8). Prestained molecular weight markers (Sigma SDS-7B), with molecular weights ranging from 32-195 kDa were electrophoresed in lane 1. The molecular weight markers were triosephosphate isomerase (32 kDa), lactic dehydrogenase (35 kDa), fumarase (52.5 kDa), pyruvate kinase (63 kDa), fructose 6-phosphate kinase (84 kDa), β-galactosidase (112 kDa) and α_2-macroglobulin (195 kDa). Rabbit anti-mouse IgG [obtained from a commercial source] (lane 2), rabbit anti-CHLDH antibody (lane 3) and rabbit anti-H_4LDH (lane 4) were all purified via protein A affinity chromatography. The purity of the anti-H_4LDH antibody compared favourably with the commercially available antibody.

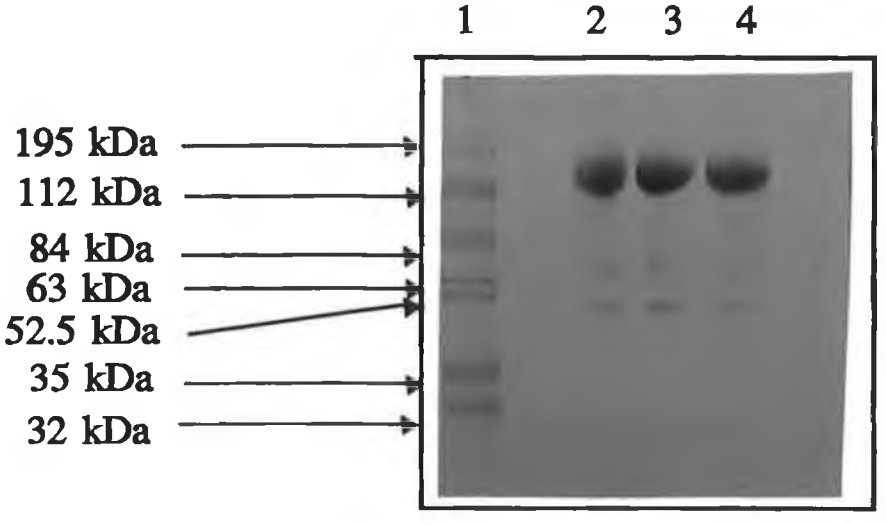
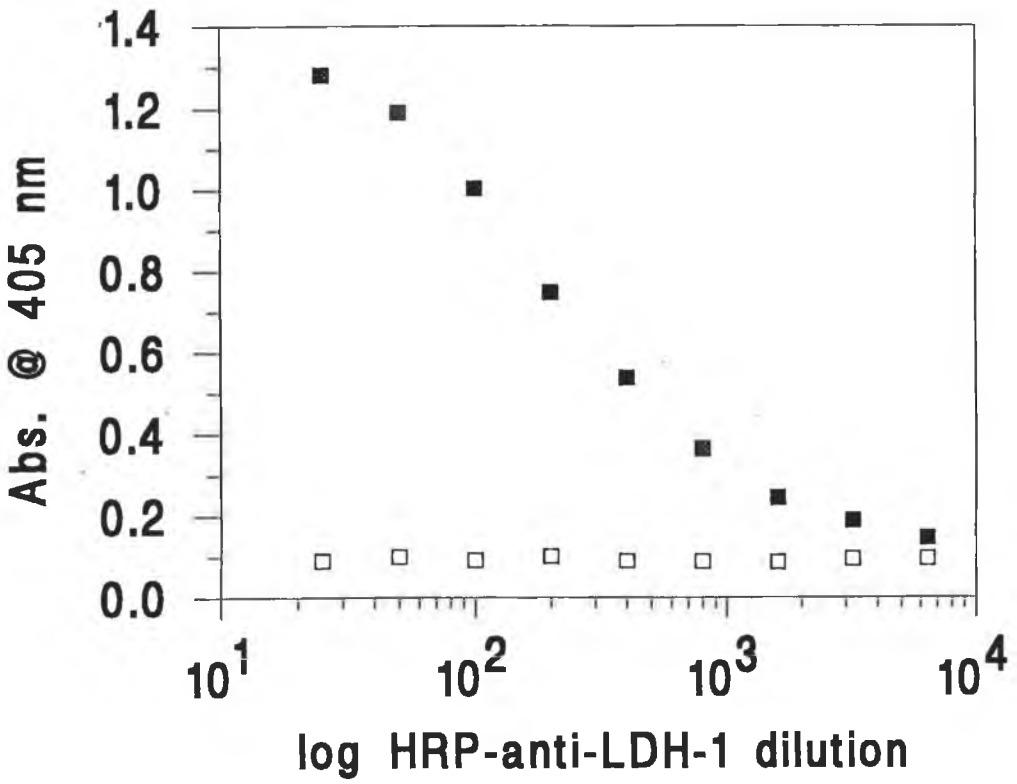


Figure 5.6.3

Determination of the working dilution of the HRP-anti-H_4LDH antibody. Serial dilutions (1:25 - 1:6400) were prepared in 3% (w/v) BSA in 0.15M PBS, pH 7.4, and titred as outlined in section 2.5.3. The working dilution was determined to be 1:100.



5.6.4 Determination of H_4LDH by two-site sandwich ELISA.

Samples of H_4LDH were prepared in 0.15M PBS, pH 7.4, at concentrations ranging from 0.003-2.500 µg/ml, and an ELISA performed as outlined in section 2.5.4. The labelled antibody, HRP-anti-H_4LDH was used at its working dilution of 1:200. The ELISA plate was blocked with 0.5% (w/v) gelatin, as there appeared to be some non-specific binding by the labelled antibody to BSA. M_4LDH (LDH-5) was the negative control. The results are plotted on a linear/log scale and the standard curve shown in Figure 5.6.4. The curve is linear across the whole range, 0.003-2.500 µg/ml (r=0.998).

5.7 Specificity of anti-H_4LDH antibody for the H subunit of LDH

A two-site sandwich ELISA was performed with 0.00 - 3.63 µg/ml H_4LDH prepared in 0.15M PBS, pH 7.4, 0.00 - 3.63 µg/ml H_4LDH in 2.00 µg/ml M_4LDH in PBS and 0.00 - 1.50 µg/ml M_4LDH in PBS. The labelled antibody, HRP-anti-H_4LDH was used at its working dilution of 1:100. Figure 5.7.1 shows that the antibody is specific for the H-subunit of LDH and does not bind to the M-subunit. No binding was observed for the range of concentrations of the M_4LDH (LDH_5). When the H_4LDH was prepared in PBS containing 2 µg/ml M_4LDH, the same curve was obtained as for the H_4LDH prepared in PBS alone. This indicates that there is no cross-reactivity with the M-subunit of LDH. The specificity of the antibody was also shown by Western blotting (Figure 5.7.2). The LDH isoenzymes were electrophoresed on a 5-20% gradient polyacrylamide gel (section 2.8.1) and blotted onto nitrocellulose paper as outlined in section 2.16. The blot yielded two strong positive bands for the H_4LDH isoenzyme and no bands for the M_4LDH isoenzyme.

An attempt was made to probe all five isoenzymes of LDH and to test the reactivity of the antibody with each one. With PAGE, all five isoenzymes run as a single band, since all five isoenzymes have similar molecular weights. The five isoenzymes can be separated, however, on the basis of the net charge on the protein. The separation is performed in 1% (w/v) agarose gels. The isoenzymes were electrophoresed in agarose gels, and blotted onto nitrocellulose paper, as for the polyacrylamide gels (section 2.16). The pore size of the 1% (w/v) agarose gels, however, is very large and diffusion of the isoenzymes occurred during the blotting procedure. Following probing with the HPR-anti-H_4LDH, no clear banding could be seen due to the diffusion of all the isoenzymes into one another.

Figure 5.6.4

Determination of the linear range of the two-site sandwich ELISA for H_4LDH. Samples (0.003 - 2.500 µg/ml) were prepared in 0.15M PBS, pH 7.4, and the ELISA performed as outlined in section 2.5.4. The second antibody, HRP-labelled anti-H_4LDH was used at its working dilution of 1:100. Absorbance values were plotted against the log H_4LDH concentration. The assay was linear across the whole range (r=0.998), indicating that the ELISA is not very sensitive.

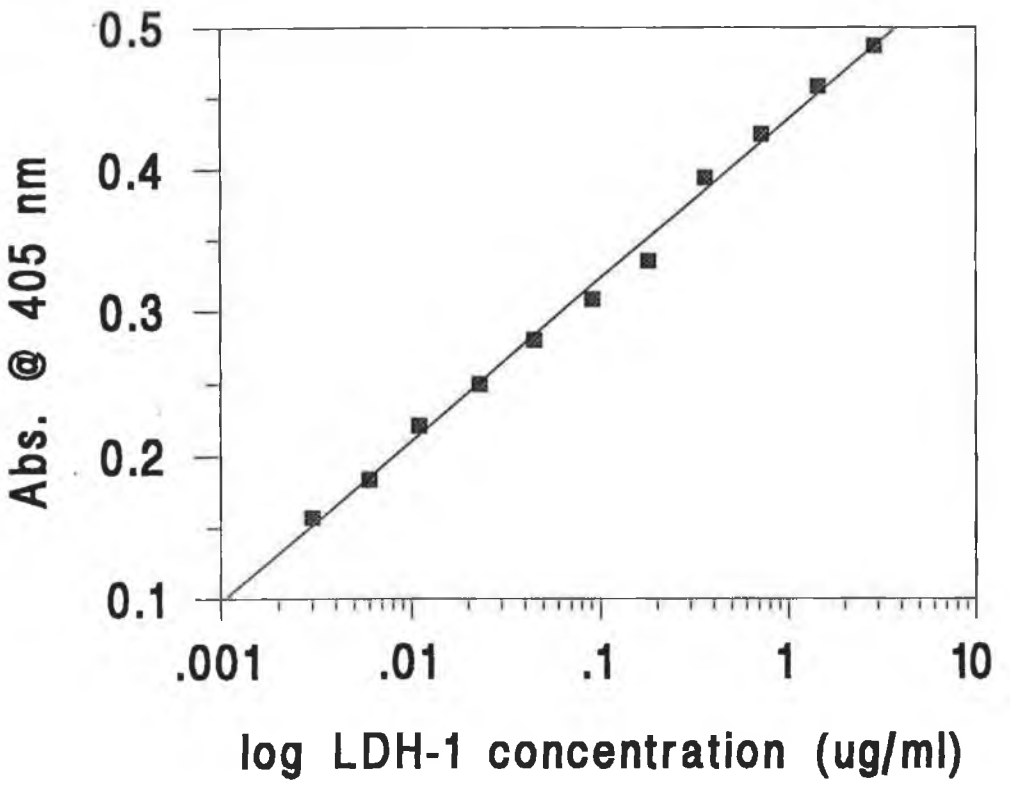


Figure 5.7.1

Specificity of the anti-H_4LDH antibody for the H subunit of human LDH. A two-site sandwich ELISA was performed with 0.00 - 3.63 µg/ml H_4LDH (□), 0.00 - 1.50 µg/ml M_4DLH (◊) and 0.00 - 3.63 µg/ml H_4LDH prepared in 2.0 µg/ml M_4LDH (■), as the antigens. No cross-reactivity of the anti-H_4LDH antibody with the M_4LDH is observed.

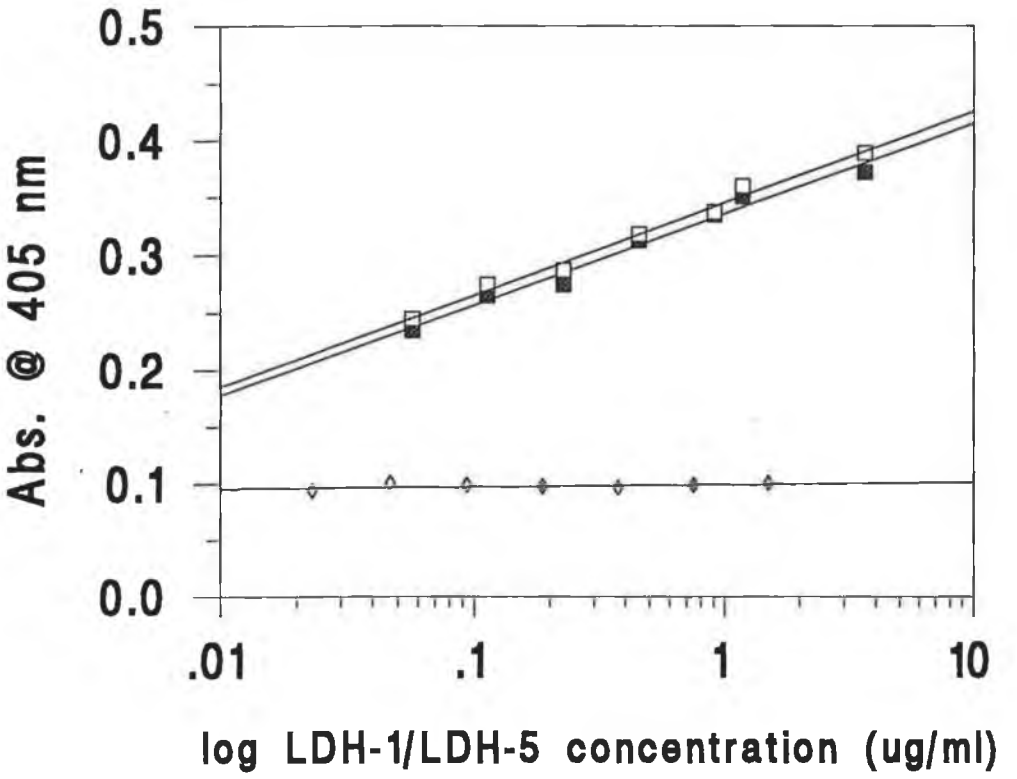


Figure 5.7.2

Specificity of the anti-H_4LDH antibody for the H subunit of human LDH, by western blotting analysis. Western blotting was carried out as outlined in section 2.16. H_4LDH (lanes 2 and 5), M_4LDH (lanes 3 and 6) and normal rabbit serum (lanes 1 and 4) were blotted onto nitrocellulose paper and probed with HRP-anti-H_4LDH antibody. Strong positive bands were obtained for the H_4LDH samples only, indicating specificity for the H-subunit of human LDH.

5.8 Isoenzyme analysis

Patient serum samples (obtained from St. James' Hospital, Dublin), with normal (<350 U/L total LDH as determined by SMAC automated analyser) and abnormal (>350 U/L total LDH), were tested in the ELISA as outlined in section 5.6.4. The antibody is specific for the H-subunit of human LDH, and so will bind to H_4, H_3M, H_2M_2 and HM_3 forms of LDH, but not the M_4 form. From the results shown in Figure 5.8.1, only one 'abnormal' sample shows an elevation of the H-subunit. For all other 'abnormal' samples, the absorbance values were in the same region as those of the normal samples. The isoenzyme profiles of all the samples tested in the ELISA (normal and abnormal), were prepared, as outlined in section 2.15. The relative percentages of each of the isoenzymes for each sample was determined after scanning densitometry. Three examples of 'normal' and 'abnormal' samples are shown in Figures 5.8.2 and 5.8.3, respectively. All the normal samples show typical normal serum isoenzyme patterns, with the ratio of LDH_1/LDH_2 less than unity. All of the abnormal samples isoenzyme profiles had an LDH_1/LDH_2 ratio less than unity, with the exception of one sample (marked with ➸), which gave a ratio greater than unity. This was the same sample which showed elevated H-subunit levels as determined by the ELISA.

Figure 5.8.1

Two-site sandwich ELISA for the H-subunit of LDH, performed on patient serum samples, with normal and elevated levels of total LDH. The samples were obtained from St. James' Hospital. Only one sample (→), with elevated total LDH, showed an increased level of the H-subunit containing isoenzymes, compared to 'normal' samples, as determined by the ELISA.

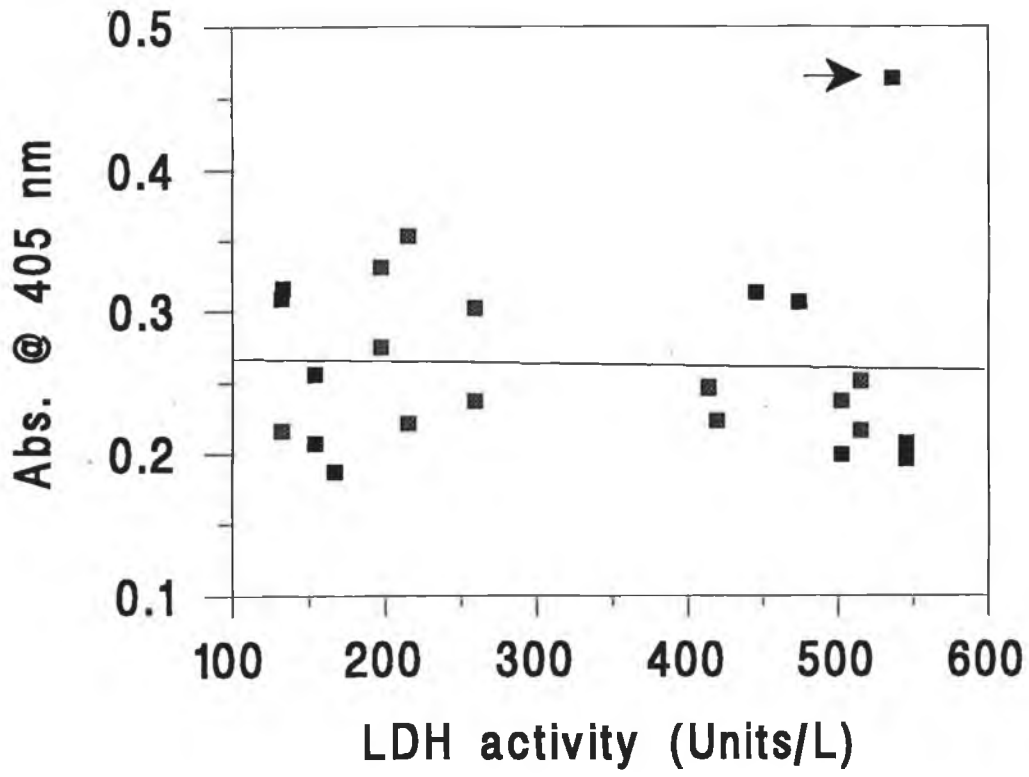


Figure 5.8.2

Isoenzyme analysis (section 2.15) of patient serum samples with normal levels of total LDH. The samples were obtained from St. James' Hospital. In all the 'normal' patient samples (3 examples of normal patient samples a, b and c are shown here), the ratio of LDH_1/LDH_2 (H_4/H_3M) was less than unity. The ratio (if greater than unity), is often used as an indicator of myocardial infarction.

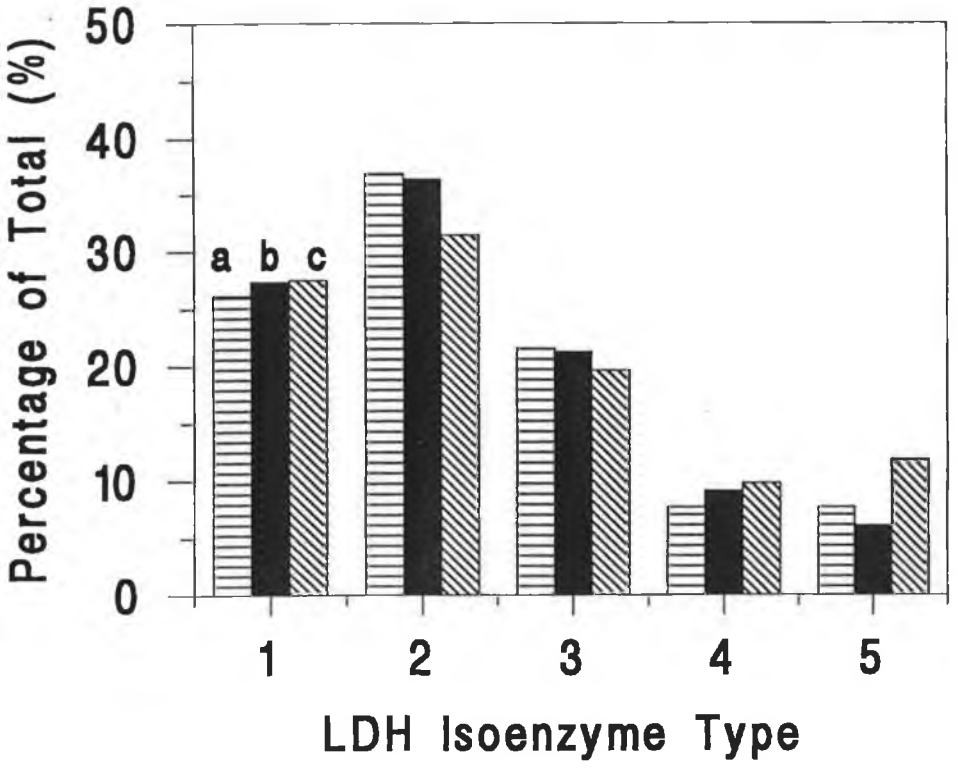
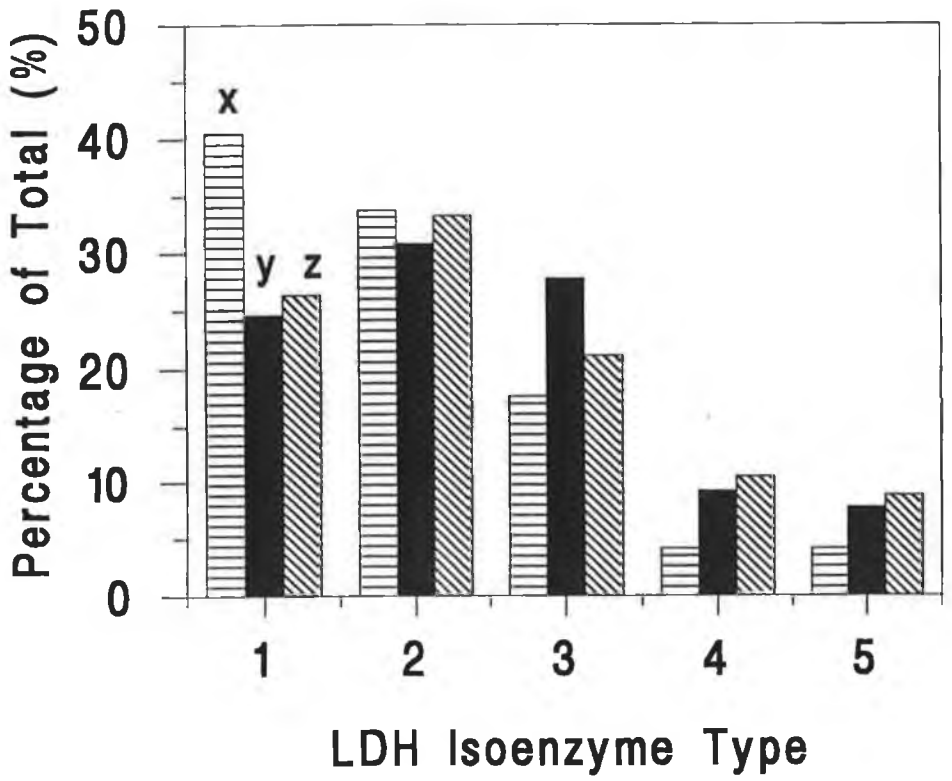


Figure 5.8.3

Isoenzyme analysis (section 2.15) of patient serum samples with elevated (abnormal) levels of total LDH. All of the 'abnormal' patient samples (three examples of patient samples with elevated LDH levels are shown here, x, y and z) with the exception of one (termed x), gave a LDH_1/LDH_2 ratio of less than unity. If the ratio is greater than unity, this is often used as an indicator of myocardial infarction. The sample (x), also gave an elevated level of the H-subunit of LDH as determined by the ELISA (Figure 5.8.1).



5.9 Discussion

Work has recently been reported on the use of microtitre plate readers in spectrophotometric enzyme assays (Butler *et al.*, 1988; Cribb *et al.*, 1989). The advantages of miniaturising assays to 96-well plate format include; speed of assay performance, economy in the use of the reagents, high throughput of samples, small sample volumes required, automatic mixing of samples and good temperature and time control.

The colourimetric assay for total LDH was miniaturised on the basis of volume reduction, while maintaining the optimal concentration of the assay reagents in the final assay mixture. The reagent concentrations and the assay conditions used were established by Gay *et al.* (1968). The determination of total LDH in serum is a weighted sum of the activities of the five isoenzymes of LDH. The portion of the total activity produced by each isoenzyme is a function of the inherent characteristics of each isoenzyme as they relate to temperature, pH, direction of the reaction, substrate concentration, coenzyme concentration, buffer and the relative amount of each isoenzyme present. The assay conditions outlined by Gay *et al.* (1968), approach optimal activity for any distribution of isoenzymes, for the forward reaction; i.e. conversion of lactate to pyruvate. This is important where the serum activity of one isoenzyme of LDH is increased, as in the case of myocardial infarction. The pH of the miniaturised assay was maintained between pH 8.5-8.6, the final lactate concentration at 45 mM and the NAD concentration at 1.5 mM. The NAD concentration for the forward reaction, should be greater than 6mM, but in the presence of the electron acceptor phenazine methosulphate (PMS) and the tetrazolium salt, 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride (INT), NAD at concentrations greater than 1.5 mM has no effect (Babson and Philips, 1965).

For miniaturisation the assay volumes were reduced while maintaining the optimal concentration of assay reagents for the forward reaction, in the final assay mixture. The total assay volume in the microtitre plate well is 245 µl. The reagents were premixed in appropriate ratios in order to simplify the reagent addition process. An eight-tip multichannel was used for rapid and accurate addition of the reagents. Timing is critical only after the addition of the 'colour reagent' (section 2.14), as the LDH remains completely inactive in the absence of NAD. One unit of LDH activity is defined as the amount of enzyme which will yield 1 µg formazan under the conditions of the assay

method. Therefore, a standard curve of formazan can be prepared and used for LDH activity determination. Alternatively, commercial serum containing known amounts of LDH can be used as standards and unknowns compared with this.

The assay used for the determination of LDH, was chosen for several reasons. It has been suggested that the forward reaction gave more accurate results, the colour produced being linear with enzyme concentration. Pyruvate, the substrate for the backward reaction is a potent inhibitor of LDH activity, when present in high concentrations, but if the pyruvate concentration is kept low, inaccuracies result when assaying serum samples with elevated LDH activity. The optimal pyruvate concentrations for enzymes of the heart, liver and serum are different. Also, the reverse reaction in the absence of a colourimetric indicator would require an ultraviolet spectrophotometer microtitre plate reader, which is not readily available in most laboratories. The assay also requires that the temperature be accurately controlled, multiple timed readings and processing of data. The simplicity and brevity of the colourimetric forward assay used made it more suitable for miniaturisation.

Polyclonal antibodies against the H_4 isoenzyme of human LDH were produced in New Zealand White rabbits. By choosing the H_4 isoenzyme, the antibodies produced were specific for the H-subunit of LDH and did not cross react with the M-subunit. The H form of LDH is a poorer immunogen than the M form. Antisera produced against the H form will generally not cross react with the M form from the same species but will crossreact with the H and M form from other species. The cross-reactivity of the antiserum was tested against heart LDH isolated from bovine, chicken and porcine sources, and the M_4 isoenzyme isolated from human muscle. The antiserum was seen to cross react to some extent with bovine and porcine LDH but not with the chicken heart LDH or the human M_4 LDH. The reason why the antiserum did not crossreact with the chicken heart LDH is probably because it is from a non-mammalian source. Serum proteins are poor immunogens in closely related species, since there is normally only minor structural differences between the same molecule in closely related species. Chickens are phylogenetically more distant from humans than pigs or cows (Catty and Raykundalia, 1988).

The antiserum was purified by Protein A (SpA) chromatography, resulting in an IgG fraction containing specific and non-specific 'host' antibodies. The ELISA developed for the measurement of H_4LDH is not very sensitive. The measurable concentration range

spans across 4 orders of magnitude, and a large change in H_4LDH concentration, only results in a small change in absorbance. The reason for the poor sensitivity could be due to low affinity antibodies, or the fact that the low titre antiserum (the H subunit is a poor immunogen), was purified by SpA chromatography. Coating plates with immunospecifically affinity-purified antibodies generally results in a more sensitive immunoassay, as there is a higher percentage of specific antibody coated to the plate. Preparation of an affinity column for purification was not feasible as the purified antigen is too expensive.

Patient serum samples with normal and elevated levels of total LDH were obtained from St. James Hospital. These samples were assayed for total LDH using an automated SMAC analyser in the Biochemistry Dept., at St. James Hospital. Normal samples had total LDH activities <350 U/L and abnormal samples had total LDH activities >350 U/L. It is expected that the purified anti-H_4LDH antibodies will bind to four of the isoenzymes of LDH containing the H subunit, i.e. H_4, H_3M, H_2M_2 and HM_3. However, in a two-site ELISA, where two antibodies, capture and second labelled antibody, must bind to the analyte, the efficiency of detecting the different isoenzymes may not be equal. Steric hindrance by the M-subunit in the HM_3 and H_2M_2 isoenzyme may result in these isoenzymes not being detected with the same efficiency as the H_4 and H_3M isoenzymes.

On AMI, H_4 (LDH_1) and H_3M (LDH_2), the predominant isoenzymes found in the myocardium, are released into the serum, resulting in elevated total LDH, flipped LDH_1/LDH_2 ratio and an elevation of LDH_1 in the serum. An increase in the serum concentration of CK-MB, an isoenzyme of creatine kinase is also observed for up to 24 hours after AMI, but it normalises again after 24 hours, and the LDH_1/LDH_2 ratio becomes important. The measurement of the LDH_1/LDH_2 ratio by electrophoretic methods was established first (Starkweather *et al.*, 1966). When this ratio exceeds 0.76, it has been reported to be 100% sensitive and 91% specific, for acute MI (Weidner, 1982). It should be noted however that the LDH_1/LDH_2 isoenzyme flip can also occur in non-AMI patients such as young pregnant females.

With the advent of immunochemical methods for the measurement of a single isoenzyme, LDH_1, several clinical studies were undertaken by various groups, to evaluate whether total LDH_1 concentration, LDH_1/LDH_2 concentration ratio or

LDH_1/total LDH concentration ratio, gave the best efficiency (the incidence of true positives to true negatives), specificity (the incidence of results below a predetermined cutoff limit among patients without myocardial infarction) and sensitivity (the incidence of results above a predetermined cutoff limit among patients with myocardial infarction), for the diagnosis of AMI. All the studies were in agreement that the determination of LDH_1 allows accurate diagnosis of AMI earlier than the ratio of LDH_1/LDH_2 (Gerhardt *et al.*, 1983; Foo *et al.*, 1981; Fogh-Andersen *et al.*, 1982). Since there is more LDH_1 than LDH_2 isoenzyme in the myocardium, sensitive quantitation of increased LDH_1 in serum would signal the existence of myocardial necrosis before the time when LDH_1 exceeds LDH_2 in the serum.

The isoenzyme profiles of all the 'normal' and 'abnormal' serum samples were prepared and analysed for a LDH_1/LDH_2 ratio greater than unity. Only one patient sample gave this result. All of the samples were also tested in the ELISA system to compare the H subunit of LDH concentration in the normal and abnormal samples. Only the sample which gave the LDH_1/LDH_2 ratio flip, showed an elevation of the H subunit over those obtained for the normal samples. No conclusion can be drawn from these results as it is not known which patients suffered from AMI. The ELISA developed does not specifically measure LDH_1 but possibly all isoenzymes containing the H-subunit. Also the ELISA is not very sensitive due to the poor antiserum obtained. An alternative method would be immunoprecipitate the M subunit, and detect the LDH_1 isoenzyme by ELISA following centrifugation. The use of monoclonal antibodies may be required to develop an immunoassay of sufficient sensitivity. LDH_1 has been quantitated to date by activity measurements after immunochemical precipitation of the M-subunit containing isoenzymes. Delanghe *et al.* (1990), stated that the catalytic properties of creatine kinase (CK), another enzyme released into the circulatory system after AMI, decreased with enzyme age, as reflected by a steady state increase in the activation energy of the catalysed reaction. They concluded that mass concentration measurement by immunochemical methods was better for infarct sizing than measurement of catalytic activity. The same may be true for LDH determination, as the rate of enzyme inactivation (half-life) is different for the different isoenzymes, and mass concentration measurements (immunoassay) of LDH_1 may be more accurate than activity measurements, after the immunoprecipitation step.

In conclusion, an ELISA for the determination of the H-subunit of LDH was investigated as a method of detecting elevated LDH_1 and LDH_2 (a consequence of AMI) in patient serum samples. The results however were inconclusive, although one clear positive was obtained. If a more sensitive immunoassay for LDH_1 was developed, using monoclonal antibodies perhaps, LDH_1 could be detected immunologically after precipitation of the four other isoenzymes. Rapid detection of LDH_1 using an evanescent wave immunosensor, as described in chapter 4, would be feasible.

CHAPTER 6

CONCLUSIONS

6 Conclusions

The research aimed to develop an evanescent wave optical immunosensor, for the determination of the clinically important diagnostic enzyme, lactate dehydrogenase. It was intended that the sensor be used for the determination of LDH in human serum samples, for the diagnosis of acute myocardial infarction. However, due to time and cost limitations, a model system using chicken heart LDH (kindly donated by Baxter Dade), was developed. This antigen was less expensive and highly immunogenic and thus allowed the production of large quantities of polyclonal antibodies.

In chapter 3, the production of these antibodies was described. A high titre antiserum (1:32,000) was raised, and the antibodies were purified by antigen-specific affinity chromatography. The antibodies were characterised for purity by size exclusion HPLC and SDS-PAGE analysis. Following labelling with HRP, a two-site sandwich ELISA for the quantitation of CHLDH was developed, which was subsequently used for the fibre ELISAs (chapter 4). The purified antibodies were also labelled with the fluorescent label, FITC, and the conjugates characterised by HPLC with PDA detection. The FITC-labelled antibodies were used in the optical fluoroimmunosensor, as described in chapter 4.

The optical immunosensor development was outlined in chapter 4. Capture antibody was immobilised onto the exposed core of optical fibres by a procedure which involved silanisation of the silica surface and the use of the heterobifunctional crosslinker, GMBS. The immobilisation procedure was optimised using ELISA techniques. The binding of antibody and antigen was probed by the evanescent wave generated at the surface of the exposed core optical fibre, via the fluorescently-labelled antibodies or antigens. The binding reaction was shown to follow pseudo first-order reaction kinetics, and so the initial slope of the binding curve was proportional to the concentration of LDH. One-step and two-step immunoassays were performed, using the same polyclonal antibodies as the capture and labelled antibodies. The lower detection limit of both assays were found to be the same, although the signals obtained from the two-step assay were higher than those obtained from the one-step assay.

Attempts were made to regenerate the fibres *in situ* in the flow cell. However, these were unsuccessful as the immunocomplex was not completely broken, by the regeneration conditions chosen, and air bubbles were introduced into the flow cell, when left *in situ*. Reproducible alignment of the fibre was also difficult, and introduced

considerable error (CV of 14.4%), into the sensor system. The effect of the sample matrix viscosity was determined, and showed an inverse relationship to the numerical aperture of the sensor probe.

The BIAcore™ monitors immunoreactions in real-time, without the requirement for reporter labels. One-step, two-step and direct (no second antibody) immunoassays were performed using this sensor. The BIAcore™ assays were found to be less sensitive in terms of the lower detection limit compared to the evanescent wave immunosensor (EWIS). However in terms of reproducibility and automation, the BIAcore™ was found to be far superior.

The effect of fluorophore-to-protein labelling ratio on the affinity of the anti-CHLDH antibody was determined using both sensor systems. As the number of fluorophore per antibody molecule increases, the affinity of the antibody for CHLDH decreased. However, for use as the labelled species in EWIS, larger signals were obtained using higher F/P ratios, up to 5 FITC/antibody molecule, thereby compensating for the reduction in affinity.

In chapter 5, a colourimetric assay for the determination of total LDH levels in serum samples was miniaturised, and was shown to correlate well with results obtained using an automated clinical analyser, based on a different reaction mechanism.

Antibodies were raised against the H subunit of human LDH. The antibodies were purified by protein A affinity chromatography, and characterised by SDS-PAGE and Western blotting techniques, for purity and specificity, respectively. The antibodies were then labelled with HRP and a two-site sandwich ELISA developed for the quantitation of human heart LDH. This ELISA was shown to be very poor in terms of sensitivity. An attempt was made to see if such an ELISA system could be used as an indicator of AMI, by measuring an increase in the H subunit of LDH in the blood. Approximately, 50 normal and abnormal (elevated total LDH) patient serum samples were screened. Only one sample showed a reversed H_4/H_3M ratio, indicative of AMI. This sample also showed elevated H subunit concentration as determined by the ELISA. However, due to the poor sensitivity of the ELISA, and the fact that only one sample out of 50 proved positive, the results were deemed inconclusive.

With a more sensitive immunoassay system, an optical immunosensor for the rapid determination of the onset of AMI could be feasible. This system would probably work best if the M subunit containing isoenzymes were first immunoprecipitated and removed

using anti-M subunit antibodies. The H_4 isoenzyme alone could then be determined using the EWIS.

CHAPTER 7

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7 Bibliography

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