THE DEVELOPMENT OF MULTIANALYTE BIOSENSORS BASED UPON PATTERNING BIOLOGICAL MOLECULES USING A PHOTOACTIVATABLE COMPOUND

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DECLARATION

The author declares that the work of this thesis has

not been previously submitted for any degree or award

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SUMMARY

The primary aim of this work was the construction of a multianalyte amperometric immunosensor. In the course of this work a new technique was developed for the selective immobilisation of organic molecules at defined areas on a variety of surfaces, including silica, glass, gold and carbon. The immobilisation technique comprised of binding avidin to the surface, and addition of photobiotin (a photactivatable biotin analogue) which bound to the avidin. This resulted in surfaces that were resistant to non-specific binding, and that allowed site specific immobilisation of biological molecules by exposure of selected areas of the surface to light, in the presence of the species to be immobilised. The application of this patterning technique was demonstrated for the immobilisation of antibodies, antigens, enzymes, lectins and nucleic acids.

In order to further demonstrate the application of this technology, a simultaneous quantitative electrochemical immunoassay was developed for the measurement of follicle stimulating hormone and luteinising hormone in human plasma. The assay showed good specificity and sensitivity, and values obtained for clinical samples correlated well with results obtained from an established commercial assay. This was the first report of an electrochemical multianalyte immunosensor for high molecular weight analytes.

Further work demonstrated the applicability of the selective immobilisation method to the construction of multianalyte enzymic biosensors and immunosensors, using ultramicroelectrodes 10 μm in diameter. It was shown that previously reported advantages of using microspot immunoassays were not applicable to these amperometric assays, as non-planar diffusion of species to ultramicroelectrodes effectively greatly increased the

instruments "field of view". Investigations into "cross-talk" between ultramicroelectrodes showed that it would be possible to fit over 350 sensors into a $1mm^2$ area.

Finally, the applicability of the immobilisation method in the detection of multiple nucleic acid sequences was also demonstrated, and an assay was developed for the detection of the Factor V_{leiden} genetic polymorphism.

The model systems described above, demonstrated both the efficiency of the protein patterning techniques developed, and its application to multianalyte sensing systems. The technology that has been developed is applicable to a generic range of sensors, and fabrication methods are compatible with the requirements of larger scale production and miniaturisation, utilising processes common in the microelectronics industry.

PUBLICATIONS ARISING FROM THIS WORK

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Contents

Figures

Tables

Γ	Density of antigen binding sites on surface
Θ	Fractional binding of antigen
Θ_{eq}	Fractional binding of antigen at equilibrium
A	Electrode area
A_e	Area of specific electrode
A_o	Area of other electodes
A_t	Total area
A & E	Accident and emergency (Casualty)
Ab	Antibody
[*Ab*]	Antibody concentration
AbAg	Antibody-antigen complex
[*AbAg*]	Antibody-antigen complex concentration
a.c.	Alternating current
Ag	Antigen
*Ag**	Labelled antigen
[*Ag*]	Antigen concentration
$[Ag]_0$	Initial antigen concentration
$[Ag]_{eq}$	Antigen concentration at equilibrium
ALP	Alkaline phosphatase
APC	Activated protein C
AST	Aspartate transaminase
BSA	Bovine serum albumin
C	Concentration (mol cm^{-3})
CCU	Coronary care unit
CK	Creatine kinase
D	Diffusion coefficient ($cm^2\ s^{-1}$)
D_{es}	Density of signal producing molecules specifically bound to electrode

D_{en}	Density of signal producing molecules non-specifically bound to electrode
D_{on}	Density of signal producing molecules bound to other electrodes
D_{nn}	Density of signal producing molecules bound to non-electrode areas
DELFIA	Dissociation enhanced lanthanide fluoroimmunoassay
DMF	Dimethylformamide
DNA	Deoxyribonucleic acid
dNTP	dinucleotide phosphate
DTSSP	3,3'-dithiobis(sulfosuccinimidylpropionate)
DUV	Deep ultraviolet
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
EMIT	Enzyme-multiplied immunoassay
F	Fractional occupancy
F	96,500C Faraday^{-1}
F_a	Apparent concentration of functional group of ligand
FITC	Fluorescein isothiocyanate
FSH	Follicle stimulating hormone
GLOD	Glutamate oxidase
GOD	Glucose oxidase
GP	General Practitioner
GRH	Gonadotrophin release hormone
hCG	Human chorionic gonadotrophin
HRP	Horseradish peroxidase
i	Current
ICSH	Interstitial cell stimulating hormone (LH)
Ig	Immunoglobulin
IgG	Immunoglobulin G
ISE	Ion selective electrode

ISFET	Ion-sensitive field effect transistor
ITU	Intensive therapy unit (Intensive care)
J	Joules
J	Mass transport flux (mol cm^{-2} s^{-1})
k_a	Adsorption rate constant
k_d	Desorption rate constant
K	Antibody affinity constant
K_d	Dissociation constant for antigen binding
LB	Langmuir-Blodgett
LDH	Lactate dehydrogenase
LFT	Liver function tests
LH	Luteinising hormone
ln	Logarithm to base e
MGIA	Microgravimetric immunoassay
MTHFR	Methylenetetrahydrofolate reductase
NAC	N-acetyl-cysteine
NFOSM	Near-field optical scanning microscope
NHS	N-hydroxy succinimide ester
NPT	Near-patient testing
NSB	Non-specific binding
Nvoc	Nitroveratryloxycarbonyl
PBS	Phosphate buffered saline pH 7.4
PCR	Polymerase chain reaction
pI	Isoelectric point
pNPP	para nitrophenol phosphate
QCM	Quartz crystal microbalance
R	Molar gas constant (8.31441 J mol^{-1} K^{-1})
RIA	Radioimmunoassay
S_{nsb}	Signal due to non-specific components

S_{sb}	Signal due to specific components
SAM	Self assembled monolayer
SAW	Surface acoustic wave
SCBU	Special care baby unit
SEM	Scanning electron microscopy
SPR	Surface plasmon resonance
STM	Scanning tunnelling microscopy
t	Time
$t_{½}$	Half life
TBS	Tris buffered saline
TDM	Therapeutic drug monitoring
TFT	Thyroid Function tests
Tris	Tris(hydroxymethyl)methylamine
TRITC	Tetramethylrhodamine isothiocyanate
TSH	Thyroid stimulating hormone
v_a	Rate of antigen adsorption
v_d	Rate of antigen desorption
x	Position

CHAPTER 1

The Road goes ever on and on
Down from the door where it began.
Now far ahead the road has gone,
And I must follow if I can,
Pursuing it with eager feet,
Until it joins some larger way
where many paths and errands meet.
And whither then? I can not say.

J. R. R. Tolkien
Lord of the Rings

1 Study Aims

The primary aim of this work was the construction of a multianalyte amperometric immunosensor, capable of quantitatively measuring two or more high molecular weight analytes simultaneously. An integrated electrochemical multianalyte immunosensor had not previously been reported in the literature, although electrochemical multianalyte immunosensing systems have been reported where more than one sensor or parts of sensors have been fabricated separately and later integrated to make an array.[1,2] In general, in the miniaturisation of sensors, it is preferable that the sensor should be constructed in such a manner that facilitates fabrication, namely from a single piece of material and employing techniques suitable for mass production.

Miniaturisation is desirable for a number of reasons including the ability to perform the analyses with a very small sample, faster response times, high throughput and reduced cost as well as the potential for implanting sensors into biological tissues.[3,4] In this study two glycoproteins, follicle stimulating hormone (FSH) and luteinising hormone (LH) were chosen as the model analytes, the measurement of FSH and LH being selected as, in clinical analysis, the determination of these hormones is usually requested simultaneously. Additionally, FSH and LH are normally assayed by heterogeneous immunoassay, the approach to be used in this work.

1.1 Biosensors:- General Principles.

Biosensors are analytical devices that use biological material to detect chemical or biological species directly, without the need for complex sample processing. Biosensors consist of a biological 'receptor' element that has the ability to bind to a very narrow range of analytes (conferring specificity) and a 'transducing' element that provides a quantitative

indication that the analyte and the biological receptor have combined. Measurement of the target analyte is achieved by selective transduction of an aspect of the biomolecule-analyte reaction into a signal. Generally, biosensors are distinguished from other biologically based analytical devices, in that the biological element is immobilised so that it is in intimate contact with, or incorporated within the transducer element. As analytical devices, they may also possess several other attractive features such as compact size, low cost, fast response time, specificity of response, simplicity in use and absence of radioactivity.[3,5]

A variety of materials have been used as the biological receptor element including enzymes,[6,7] antibodies,[8] antigens,[9] organelles,[10] cell membrane receptors,[11] complete cells[12] and slices of tissue.[13,14] Of these biological materials, enzymes and antibodies are the most frequently employed, which is a reflection of their widespread use in conventional analytical techniques. One great advantage of the use of biological molecules is that they possess discriminatory powers equal to or better than the most powerful of the non-biological analytical techniques.[15] For example, both enzymes and antibodies are able to distinguish between such closely related molecules as optical and geometrical isomers. An additional advantage of the use of biological molecules in analyses, is that it may be possible to incorporate a functional amplification system, which can allow improvement of both detection limits and sensitivity by several orders of magnitude.[16]

The transducer element of a biosensor detects a change in physiochemical properties upon binding of the analyte to its biological receptor. This interaction may result in a change of pH, electron transfer, refractive index, heat transfer, or uptake or release of specific ions or gases. Transducer mechanisms include electrochemical,[6,17] optoelectronic,[18] piezoelectric[19]

and thermometric[20,21] devices. Changes in physiochemical properties and possible detection methods used in biosensors are summarised in Table 1.1

Physicochemical Change	Possible Detection Methods
Heat	Calorimetric
Optical	Absorbance, luminescence, fluorescence, reflective, ellipsometric, Surface Plasmon Resonance, optoelectronic
Electrons	Amperometric
Ions	Potentiometric, conductimetric
Gases	Amperometric, potentiometric, piezoelectric, conductimetric
Mass	Piezoelectric

Table 1.1. Physicochemical changes and possible detection methods in biosensors.

1.1.1 Electrochemical transducers

Electrochemical biosensors combine the analytical power of electrochemical techniques with the selectivity and specificity of biological molecules. Clark and Lyons[22] first introduced the concept of the "soluble enzyme" electrode, but the earliest incorporation of an enzyme into an electrode was by Updike and Hicks,[6] who immobilised glucose oxidase in a gel over an oxygen electrode to measure the glucose concentration of biological fluids. The majority of electrochemical biosensors can be classified into one of two groups; amperometric or potententiometric devices. In amperometric devices[5] an electrical potential is applied to an electrode system, which facilitates reduction or oxidation of electroactive species at the electrode surface. This "redox" reaction involves the transfer of electrons between the electrode and an electroactive species, with a current being produced which is proportional to the concentration of the electrochemical species at the electrode surface.

Amperometric transducers have been very widely used in biosensing, particularly systems based upon the detection of oxygen or hydrogen peroxide. The amperometric approach has high sensitivity and a linear concentration dependence (compared with a logarithmic relationship in potentiometric systems), and improved selectivity can be obtained by careful selection of the electrode potential. Many molecules (such as proteins) are not intrinsically electroactive, therefore it is frequently necessary to introduce enzymic labels that can catalyse redox reactions and produce electroactive species. For example, amperometric sensing of immunological reactions can be performed by using an immobilised antibody to bind the analyte, and then adding a second enzyme labelled antibody which is also directed against the analyte.[23-30]

The first potentiometric enzyme electrode was reported by Guilbault and Montalvo[7] for the determination of urea. In this technique, the fact that the Nernst potential across a membrane was dependent upon the concentration of electroactive species on either side of the membrane was exploited; thus if the concentration on one side was kept constant then the potential generated across the membrane (measured against a reference electrode maintained at zero current flow) was proportional to the concentration on the other side (the concentration - potential relationship being logarithmic). The use of ion-selective membranes introduced specificity into the system. Examples of potentiometric sensors are ion-selective electrodes (ISEs) including the pH electrode[31] and ion-sensitive field effect transistors (ISFETs).[32] Potentiometric electrodes have been used as the transducer in biosensors employing immunological reactions, employing a number of approaches,[33-36] one of which, is to measure the change in potential caused by the alteration in the ionophoric properties of an immobilised antigen (either ionophoric in its own right, or conjugated to an ionophore) when selectively bound by antibodies.[37,38] Due to their small size, their generic

technology and ease of manufacture ISFETs are very attractive in biosensor technology, one example of their usage is in detecting changes in charge densities and isoelectric points that take place upon the formation of an antibody-antigen complex.[39]

A third electrochemical transduction system that has been used in biosensors is the measurement of conductance. An example is the measurement of urea using urease which results in the production of NH_4^+ causing an increase in the conductance of the solution.[17] Conductimetric biosensors utilising immunological reactions for the measurement of pesticides[40] and drugs of abuse[41] have also been developed. The use of conductance has not been widely used in biosensors, partially because conductimetric techniques tends to suffer from poor specificity of measurement as the resistance of a solution is dependent upon all of the ions present, and there are variations in ionic strength and buffering capacity between samples.

1.1.2 Optical Transducers

The measurement of absorbance or emission of electromagnetic radiation, by either reactants or products of a biological system has been widely used in biosensors. Optical transducers are able to respond to changes in absorbance, fluorescence, luminescence, reflectance, resonance, interference or refractive index. Early optical sensing techniques generally required the use of labels, and these indirect optical sensing techniques are still very widely used in biosensor technology. In some of the earliest optical systems, reactants were immobilised in columns or on tubing and optical readings (such as absorbances) were taken downstream in a flow through system.[42] Fibre-optic technology enables the fabrication of "optrodes", where the reagent phase is immobilised on a single optical fibre, so that changes in optical properties of the reagent phase attributable to the analyte can be

measured. Antibodies can be labelled with a number of materials in order to allow optical measurement. Enzymes can be used as a label to eliminate substrates or generate products that absorb light,[43-47] fluoresce[48] or luminesce,[49-51] and these have been widely used in biosensor technology. Antibodies can also be directly labelled with fluorescent compounds[52] or coloured material such as red blood cells[53] or colloidal gold.[54]

Optical techniques for the direct monitoring of changes in adsorption, fluorescence, refractive index or light scattering have been used in biosensors. These direct optical sensing techniques (those that do not require a label) include attenuated total internal reflection,[55] ellipsometry,[56] surface plasmon resonance[57] and waveguides.[58] In most of these systems, light entering the device is directed towards the sensing surface and then reflected back, with the light emerging from the device revealing information regarding the events occurring at the sensor surface. These direct sensing techniques are particularly useful for real time applications such as investigating reaction kinetics, although they tend to be affected to a greater extent by non-specific interference than do indirect sensing techniques. Attenuated total internal reflection,[59] ellipsometry,[60] surface plasmon resonance[61] and waveguides[62] have all been used to monitor immunochemical reactions.

1.1.3 Mass Detecting transducers

In 1880 Jacques and Pierre Curie discovered that a mechanical stress applied to the surface of quartz (and a number of other crystals) resulted in an electrical potential across the crystal; the piezoelectric effect, the magnitude of which was proportional to the mechanical stress applied.[63] The piezoelectric effect only exists in materials that crystallise in noncentrosymmetric space groups. The quartz crystal microbalance (QCM) makes use of this phenomenon, and comprises a thin quartz crystal sandwiched between two electrodes. An alternating current (a.c.) field is established across the crystal causing vibrational motion

of the crystal at its resonant frequency. One of the parameters that the resonant frequency is sensitive to, is a change in the mass of absorbate on the crystal. In piezoelectric biosensors, crystals are coated with an adsorbent that selectively interacts with the analyte of interest, subsequent binding increases the mass of the coated crystal and alters its frequency of oscillation. Monitoring the change in oscillation frequency enables determination of the change in mass, which is proportional to analyte concentration. However, in thick films of adsorbed layer (as is generally the case in biosensing), the change in resonant frequency is not only caused by the addition of mass, but by change in the viscoelastic response of the sensor system.[64] Thus, crystals employing thick elastic layers which "couple" into the supporting fluid medium (e.g. multilayer sensors containing organic molecules), which makes interpretation of data difficult.

Notwithstanding this, the QCM has excellent sensitivity, being capable of measuring mass changes corresponding to submonolayer adsorption and desorption. Consequently it has been employed in the analysis of gaseous environmental pollutants,[65-67] herbicides,[68,69] medical diagnostic assays[70,71] and in monitoring antibody-antigen interactions.[19]

Surface Acoustic Wave (SAW) devices have also been used as transducers in biosensors. In these devices, an acoustic wave is generated by the application of an alternating voltage across an interdigital transducer (a pattern of interlaced metal electrodes). The acoustic signal produced is detected by a second interdigital transducer located a few millimetres away. The adsorption of analyte to the crystals slows the acoustic wave, and the change in velocity (which is proportional to the analyte concentration) is recorded. Again SAW devices have seen application in the analysis of gaseous environmental pollutants,[72] and in monitoring antibody-antigen interactions.[73]

Surface plasmon resonance (SPR) is a quantum optical-electrical phenomenon arriving from the interaction of light with a suitable metal or semiconductor surface, where under certain conditions the photon's energy is transferred to packets of electrons called plasmons.[57] In SPR the angle of incident monochromatic light is varied. At most angles the light is reflected but at a particular angle the plasmons are excited and the incident light is almost completely absorbed. The angle of incidence that causes resonance is dependent upon the refractive index of the metal, the nature of the metal's surface (i.e. roughness) and the refractive index of the medium in contact with the metal surface, therefore changes in mass at the surface result in a change to the resonance characteristics.

A disadvantage of direct immunosensing techniques (e.g. QCM, SAW and SPR) is that any protein that is adsorbed will interfere with the assay, whilst for indirect immunosensing techniques, only proteins that contribute to the signal as generated by the label will interfere with the assay (unless the non-specific binding interferes with the molecular recognition between immobilised molecule and analyte).

1.1.4 Calorimetric Transducers

Calorimetric biosensors are constructed by attaching the biological component to a thermistor, which detects the heat generated by enzymatic reactions.[74,75] These devices have been used for enzymatic measurements[76] and have also been used for immunological analysis by labelling antibodies with enzymes.[77,78]

1.2 Immunosensors

The primary aim of this work was the construction of a multianalyte amperometric immunosensor. Immunosensors are biosensors where the biological receptor component immobilised at the transducer is an antigen or an antibody. Immunosensors set out to perform an immunoassay without the requirement for the multiple, time-consuming, procedures that are usually associated with this technique. Immunoassay may be defined as a technique based on the reaction between an antigen and an antibody for measuring the concentration of either reactant in solution. In solid phase immunoassay, the antibody or the antigen is immobilised on a solid insoluble matrix.[79] An indirect immunosensor uses a separate labelled species that is detected after binding, e.g. enzyme linked immunosorbent assay (i.e. a heterogeneous immunoassay), whilst direct immunosensors can monitor binding in real time, by measuring parameters such as changes in mass or optical properties such as refractive index. Immunosensors have been used for a number of applications in medical, environmental and biotechnology measurements, some of which are summarised in Table 1.2.

1.3 Principles of immunoassay

1.3.1 Solid Phase Immunoassay

Antigens and antibodies can be coupled to an insoluble matrix with the retention of immunological activity,[79] which facilitates the separation and washing steps required to differentiate bound and free fractions of the label in heterogeneous assays. The chemical composition and the form of the solid phase of the immunoadsorbent can be varied in many different ways, solid phases that have been used include fine powder particles,[82] thin discs,[83] magnetic particles,[84,85] microtitre wells[86] and coated tubes.[87]

Transduction technique	Medical Applications	Environmental applications
Amperometric	hCG,[23,29] LDH5,[30] theophylline,[24] von Willebrand Factor,[25] apolipoprotein E,[26] α1-acid glycoprotein[27]	Herbicides[28]
Potentiometric	Penicillin,[33] Insulin,[34] hCG,[35] HBsAg[36]	
Conductometric	Methamphetamine[41]	Pesticides[40]
Absorbance	hCG,[44] C-reactive protein,[45] theophylline,[46] insulin[47]	
Fluorescence	β2 microglobulin[48]	
Luminescence	Oestradiol,[49,50] α-interferon,[49,50] hCG,[81] IgG[49,50]	Mercury[51]
Ellipsometric	γ interferon[60]	
SPR	hCG[61]	
Waveguides	Microbial toxin[62]	
Interferometric	HBs Ag[80]	
Piezoelectric	Drugs of abuse,[70] HIV antibodies[71]	Gaseous pollutants[65, 66] herbicides[66,69]
SAW	IgG[73]	Gaseous pollutants[72]
Calorimetric	Proinsulin,[77] albumin,[78] gentamicin[78]	

Table 1.2 Examples of applications of immunosensors for medical and environmental monitoring.

1.3.2 Antibody-antigen binding.

Antibody-antigen binding is due to the summation of a number of relatively weak non - covalent forces including Coulombic interactions, van der Waals forces and hydrophobic and hydrogen bonding.[88] The binding of an antigen (*Ag*) to the antibody (*Ab*) is reversible and the strength of the interaction is dependent upon the affinity of the antibody and the number of binding sites on the antigen. Antibody-antigen binding can be expressed as an equilibrium as shown in Equation 1.1

$$Ab + Ag \Leftrightarrow AbAg \quad (1.1)$$

The equilibrium point is dependent upon the concentration of the antibody and antigen ([*Ab*] and [*Ag*]) respectively and upon the strength of their interaction. This strength of interaction can be expressed as an affinity (or association) constant, K_a (Equation 1.2). Values for this constant range between 50 and 10^9 M^{-1}.[89]

$$K_a = \frac{[AgAb]}{[Ab][Ag]} \quad (1.2)$$

The affinity is independent of the number of binding sites on the antigen. Avidity is the term used to express the total binding strength of the antibody to all of the binding sites on the antigen. A typical IgG molecule binds at least four orders of magnitude more strongly to a multivalent antigen if both binding sites are engaged, than if only a single site is bound.[89] Antibody-antigen reactions at surfaces are discussed more fully in Chapter 4.

1.3.3 Labels in Immunoassay

Early immunoassay techniques used radioactive labels to quantify the degree of binding. The first of these techniques to incorporate radiolabels was radioimmunoassay (RIA) which was developed and applied to measurement of substances in humans by Berson and Yalow.[90,91] In recent years there has been a move away from the use of radioactive labels, and they have largely been replaced by amongst others luminescent,[92] fluorescent,[93] and

enzymatic labels.[94,95] These non-isotopic labels possess a number of advantages such as longer shelf life, shorter assay time, easier automation and improved detection limits. They also introduce the possibility of developing disposable devices that can be used to perform near-patient testing.[96,97] The main objective of this study was to establish a multianalyte immunosensor based upon the electrochemical detection of the formation of products catalysed by enzymic labels. Immunoassays utilising enzyme labels are generally classified into one of two classes, heterogeneous and homogeneous assays.

1.3.4 Enzyme Immunoassay

In heterogeneous enzyme immunoassay, after the antibody and antigen have been incubated together, a step is required to separate the free antigen and antibody from the antibody-antigen complexes. The enzymic activity of one or both of these fractions is then assessed. The most common heterogeneous enzyme immunoassay is Enzyme-Linked Immuno-Sorbent Assay (ELISA), in this technique either the antigen or the antibody can be labelled with the enzyme. In the so called 'Sandwich' ELISA assay (Figure 1.1), an excess of immobilised antibody is incubated with the sample (Figure 1.1a), and the antigen binds with the immobilised antibody. After washing away the unbound sample constituents (Figure 1.1b), the immobilised antibody-antigen complex is incubated with excess enzyme-labelled antibody. This enzyme labelled antibody binds to a second antigenic site (Figure 1.1c). Excess enzyme-labelled antibody is washed away. Therefore, the remaining enzyme activity is directly proportional to the antigen concentration (Figure 1.1d).

In homogeneous enzyme immunoassays (enzyme multiplied immunoassay technique (EMIT)), a separation process is not required. In this technique antigen-coupled enzyme activity changes when bound to an antibody. Upon the addition of a sample, less

antigen-enzyme conjugate will bind to the antibody, as it is in competition for the limited numbed of antibody binding sites with free antigen from the sample. Therefore, when antigen from the sample is added to the reaction mixture, the change in enzymic activity is inhibited, and the degree of change is inversely proportional to concentration of the analyte in the sample. ELISA is generally applicable to the measurement of almost any antigen, and can detect substances at much lower concentrations than can EMIT. EMIT is mainly used for the analysis for substances of relatively low molecular weight, including many drugs.

1.4 Applications of Biosensors

Biosensors have applications in a wide variety of fields including defence,[98] safety,[99] agricultural and industrial analysis,[100] process monitoring,[101] environmental monitoring,[102,103] food monitoring,[104] drug screening[105] and medical diagnosis and monitoring.[106] Commercially, biosensors have not as yet achieved as significant percentage of sales in the diagnostics market as in some predictions, but there is the potential of hugely increased sales.[107] Specific examples of the applications of immunosensors are shown in table 1.2.

1.4.1 Biosensors in Clinical Chemistry

The use of biosensors in medical diagnosis and assessment introduces an increased capacity for decentralised testing, i.e. performing the analyses outside of the central laboratory. Dependent upon the analyte this can be important in special care units such as intensive therapy (ITU), cardiac care (CCU) and special care baby units (SCBU), on hospital wards, outpatient clinics, General Practitioner's surgeries or even for patient self-monitoring at home. The main criterion for determining if decentralised testing is effective, is whether obtaining the result immediately will aid in the treatment of the patient, other factors are whether a reduction in cost or inconvenience will result. Sensors have a greater potential for

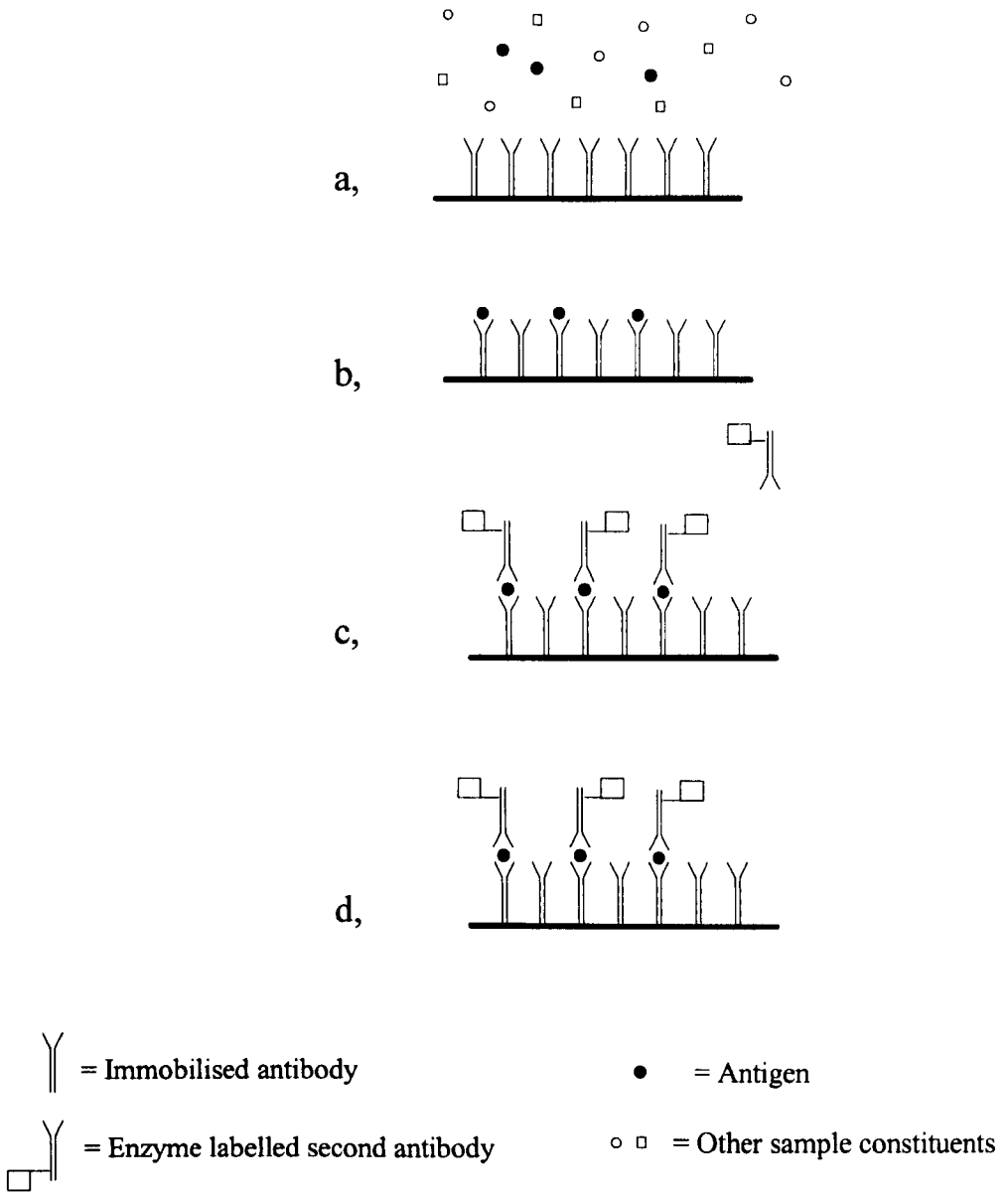

Figure 1.1. Schematic representation of the ELISA 'Sandwich' assay. An excess of immobilised antibody is incubated with the sample (a), and binds the antigen therein. After washing away the unbound sample constituents (b), the immobilised antibody-antigen complex is incubated with excess enzyme-labelled antibody. This enzyme labelled antibody binds to a second antigenic site (c). Excess enzyme-labelled antibody is subsequently washed off, and the remaining enzyme activity is directly proportional to the antigen concentration (d).

the de-skilling of analysis than of any previous technology.[108] Table 1.3 shows frequently requested tests from various sources. Whilst this list is clearly not definitive, it illustrates potential applications of biosensors for near patient testing.

It can be seen from Table 1.3, that only a small proportion of the frequently performed assays on units where the patient is critically ill (i.e. ITU and SCBU) are amenable to assay using biosensors. By contrast, biosensors can play a much more important role in accident and emergency (A&E) departments, where their ease of use and rapid response time can be invaluable in diagnosis, particularly in the case of poisonings. However, the speed of obtaining a result is not only important in life or death situations. In tests performed on outpatients (in clinics, GP's surgery and patient self-testing), a rapidly obtained result may assist in the treatment of the disorder. Examples of this are self-monitoring of blood glucose by diabetics, who can then adjust insulin dosage and/or diet accordingly, and therapeutic drug monitoring where circulating levels of the drug are determined, such that if necessary the dosage of the drug can be changed so as to bring the circulating levels within the effective range. In addition, obtaining a result rapidly, can obviate the need for extra visits, saving time and money for both the patient and the health service.

Near-patient testing (NPT) is not without problems, as errors can occur in the operation of even the most user-friendly tests and unskilled or semi-skilled staff are often unaware of these and thus they may go unnoticed. The risk of this occurring can be reduced by skilled laboratory staff providing proper training and support and the introduction of a Quality Control System.[109]

ITU	SCBU	CCU	A&E	General Wards
pH	as ITU	K+	as ITU	Na+
pO2	plus	LDH *	plus	K+
pCO2	Bilirubin	CK *	Paracetamol *	Urea *
Na+	Intralipid	AST *	Salicylate *	Creatinine *
K+			Ethanol *	Glucose *
Cl-			Amylase*	Uric acid *
HCO3-			Uric acid *	
Urea *			Drugs of abuse *	
Creatinine *				
Glucose *				
Total Protein				
Albumin				
Calcium				

Out-patient clinics	GP.s surgery	Patient Self monitoring	Screening Tests
Glucose *	as Outpatient clinics	Glucose *	alpha-foeto protein *
Urea *	plus	TDM *	Phenylalanine *
Creatinine *	hCG *		TSH *
Cholesterol *	LDH *		
Triglyceride *	CK *		LFT = Liver function tests (mainly enzyme assays)
LFT *	AST *		TFT = Thyroid function tests (hormone assays)
TFT *			Infertility Tests are mainly hormone assays
Infertility Tests *			TDM = Therapeutic drug monitoring
Uric acid *			
TDM *			

Table 1.3. Some of the most frequently requested clinical chemistry investigations from different sources. Assay marked * are those frequently performed utilising functionality of biological molecule.

At present most biosensors employ the biological functionality of enzymes or antibodies to impart specificity to the analytical system. The greatest market in biosensors for medical applications is in devices for the self-monitoring of glucose by sufferers of diabetes mellitus. Self-monitoring allows diabetics to better control glucose levels by adjusting their diet and/or insulin dosage in response to the results. It has been demonstrated that at least in some diabetic groups improved control of glucose concentration results in a better prognosis, with a reduced risk of the disease's complications such as peripheral neuropathy and retinopathy.[110]

The measurement of glucose provides a good example of the varying degrees of complexity that are possible in biosensor technology. These biosensors are based upon the concentration of glucose being the rate limiting parameter in an enzyme catalysed reaction, the enzyme employed usually being glucose oxidase (GOD), which can be obtained as large amounts of a pure preparation relatively cheaply. The simplest of these sensors are the so called "stick tests." In these glucose acts as the substrate for GOD resulting in the formation of hydrogen peroxide, which oxidises a chromaphore resulting in a colour change. The resultant colour intensity is proportional to the glucose concentration which can be determined by comparison of the colour with a chart or by the use of a simple reflectance spectrophotometer.[111,112]

A successful commercial product for the determination of glucose is the Exactech device marketed by Abbott (Chicago, IL, USA). In this device GOD oxidises glucose to gluconolactone and in the process is itself reduced. GOD is reoxidised by means of a mediator, which in the process of being reoxidised at an electrode surface results in the

production of a current, proportional to the glucose concentration. This process is illustrated in Figure 1.2.

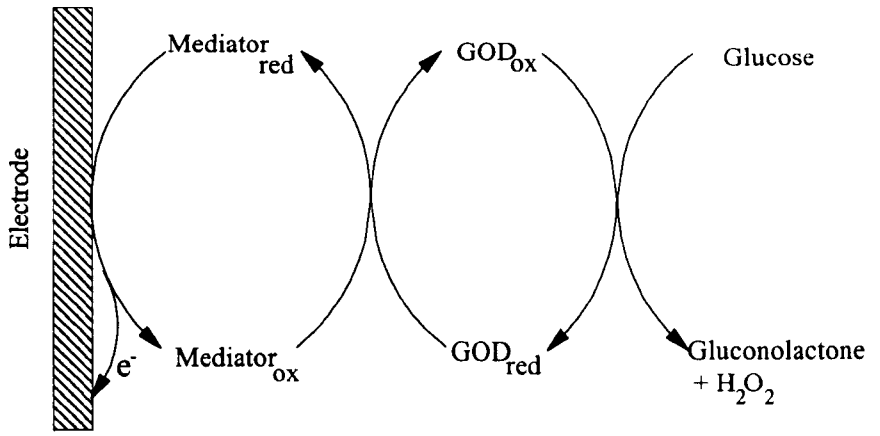

Figure 1.2. Scheme for amperometric measurement of glucose using glucose oxidase and a mediator. Glucose oxidase (GOD) oxidises glucose to gluconolactone and in the process is itself reduced. GOD is reoxidised by means of a mediator, which in the process of being reoxidised at an electrode surface results in the production of a current, proportional to the glucose concentration.

A glucose biosensor can be incorporated into more complex instruments which are capable of measuring several analytes simultaneously.[113] Work is in progress to incorporate biosensors into implantable artificial organs to provide feedback loops, for example an *in vivo* glucose biosensor connected to an insulin pump has the potential of acting as an artificial pancreas, maintaining glucose homeostasis in diabetics.[114]

For sensors employing antibodies as the biological element providing analytical specificity, a number of qualitative assays based upon the visual observation of agglutination reactions are on the market. Most common are tests for human chorionic gonadotrophin in urine which is an indicator of pregnancy.[115] These types of tests are discussed more fully in Chapter 3.

1.5 Multianalyte Biosensors

As already mentioned individual biosensors can be incorporated into more complex instruments which are capable of measuring several parameters simultaneously.[113] A major problem in the construction of a multianalyte biosensor fabricated as a single element, is the selective deposition of different biological molecules at predetermined sites (discussed in detail later in this chapter). A number of enzyme based multianalyte biosensors have been reported, using a variety of protein immobilisation methods.[116-119] These multianalyte sensors have been employed for the simultaneous determination of small organic molecules of biological importance such as glucose, urea and galactose. A single electrochemical multianalyte immunosensor had not previously been reported, although electrochemical multianalyte immunosensing systems have been reported where several sensors or parts of sensors have been fabricated separately and joined to make an array.[1,2]

Existing multianalyte immunosensors tend to be qualitative assays, examples of such systems are the Triage™ test for qualitative determination of drugs of abuse,[120] and the Spectral Diagnostics assay for markers of myocardial infarction.[121] These assays are based upon immunochromatography reactions, which are observed visually and are generally unsuitable for quantitative assay. Multianalyte immunosensors using optical transduction have also been reported,[122,123] based upon glass optical waveguides or fibre-optics, where a change in absorbance or fluorescent intensity is related to the concentration of a labelled antibody close to the transducer surface, and therefore to the presence of antigen at the surface.

Simultaneous multianalyte immunoassays, where several analytes are measured by a simple procedure, are required in a number of clinical situations, such as the measurement of

hormones related with thyroid function and the measurement of gonadotrophins for the investigation of infertility.

1.6 Protein Patterning on Sensor Surfaces

An important requirement for the success of this study was the development of a method for the specific immobilisation of biochemical species at individual electrodes within an array. A problem in trying to specifically immobilise species on one electrode in an array, is in preventing non-specific adsorption of the protein to the other electrodes and the surrounding area, as the majority of proteins will adsorb very strongly to a variety of surfaces.[124,125] Prevention of non-specific adsorption to the area surrounding the electrode is particularly important when using microelectrodes, as there is an increased diffusion to the electrode from the surrounding area with these very small electrodes when compared to macroelectrodes (see Chapter 4).

Most proteins are highly surface active, that is they have a strong tendency to accumulate at interfaces. Proteins usually have a hydrophilic surface with a hydrophobic inner, thus they tend to "unfold" on hydrophobic surfaces or stick to hydrophilic surfaces. A number of factors play a role in the adsorption process; hydrophobic interactions with non-polar surfaces, electrostatic interactions between the protein and the surface, surface heterogeneity, the solvent used, other species present in solution and the structure and possible denaturation of the protein.[126]

Protein adsorption is frequently irreversible (often due to denaturation as a result of the protein unfolding), but an adsorbed protein, may be displaced by another (usually larger) protein even if the adsorbed protein does not desorb in pure solvent. Protein adsorption tends to the formation of a monolayer (although its structure may be complex) as the

process involves the loss of some of the molecules conformational and hydration energy (which is thermodynamically favourable) when it encounters an interface.[125] The result is that at the interface, the protein tends to undergo a conformational change from their globular conformation to an extended chain conformation. Protein multilayers tend not to form because, as protein is deposited on the surface, the interface becomes less distinct and it becomes less thermodynamically favourable for subsequent molecules to undergo changes in conformation and hydration. Relatively simple thermodynamic descriptions such as the Langmuir Isotherm[127] can not readily be applied to protein adsorption, as it is a complex phenomenon involving a number of processes that occur simultaneously.[124,128] These processes include structural alterations of molecules at the interface and interactions with other adsorbed molecules. A number of more complex descriptions of protein adsorption kinetics have been suggested.[124,126,128]

1.7 Non-specific Binding

Non-specific binding (NSB) is an inherent property of proteins (including antibodies) and is considered the major obstacle to the development of immunosensors.[129] In solid-phase enzyme immunoassays for single analytes, non-specific binding can be caused by three mechanisms. In the first of these mechanisms, which will be referred to as "direct reagent binding," the second antibody-enzyme conjugate binds to the surface of the solid support. This type of binding results in elevated signals that are usually relatively unaffected by the sample composition. Direct reagent non-specific binding results in a diminished ability of the assay to distinguish the "true" analyte derived signal from the elevated background signal, resulting in a poorer assay sensitivity.

The second mechanism which will be referred to as "sample mediated binding," is where the antibody-enzyme conjugate binds to the solid support via a sample derived species other

than the analyte. If this "cross-linking" species is present in equal quantities in all samples (including standards), then the effect will be similar to that observed for direct reagent binding, i.e. there is a diminished ability of the assay to distinguish the "true" analyte derived signal from the elevated background signal. If, however, the "cross-linking" species is present in differing quantities in any samples, variation of the observed signal will occur unrelated to the analyte concentration, resulting in a reduced assay specificity.

The third mechanism is "direct sample binding." This is where enzymes catalysing the same reactions as those catalysed by the enzymes conjugated to the antibodies, bind to the surface of the solid support. Direct sample binding results in poorer assay sensitivity and specificity. In multianalyte sensing systems there is a fourth type of non-specific binding; "capture antibody non-specific binding." This occurs where a capture antibody binds non-specifically to a sensor element designed for the analysis of a different antigen. This "inappropriately" bound antibody is then able to bind its antigen, and subsequent binding of a labelled second antibody can occur. This results in poorer assay specificity, and can easily lead to the production of incorrect results.

Prevention of non-specific binding is of great importance in developing a technique for immobilising organic molecules (including biomolecules) in a predetermined pattern on a surface. Non-specific adsorption of reagents or of sample components, may lead to interference and incorrect results in the assay. The techniques for reduction of non-specific adsorption are generally based upon either, coating the surface with a substance that will decrease the tendency of proteins in solution to bind to the surface, or to mask areas of the surface with a substance that can be stripped off along with any protein, leaving the bare surface exposed. A number of approaches that have the potential to solve this problem have

been reported in the literature including, pre-coating the electrode surface with protein or amino acids,[125] and using electrochemical adsorption and desorption of thioalkanes and thioalcohols.[130] These techniques are discussed in greater detail later in this chapter.

1.8 Immobilisation

A large number of techniques have been employed to immobilise the biologically active moiety of electrochemical biosensors on or in close proximity to the electrode. These can be categorised as encapsulation, entrapment, adsorption, cross-linked adsorption, electrostatic attraction, covalent attachment and affinity binding as illustrated in Figure 1.3.

1.8.1 Desirable properties of immobilisation method.

An immobilisation technique should not adversely modulate activity of the biological molecule, and should not limit access of non immobilised reactants to the immobilised molecule. This is particularly important where reactants of relatively high molecular weights (e.g. antigens) are involved. In addition, there is a requirement for an efficient sensing surface i.e.; a high surface density of functional molecules, the absence of non-specific binding, stability and durability. Several parameters such as pH, ionic strength and the surface chemistry of the solid surface influence the amount of immobilised protein at a solid surface. These parameters can be expected to influence the orientation and conformation and thereby the biological activity of the protein.[131,132]

In immunosensors with high molecular mass analytes, those methods that immobilise the antibody by trapping it by virtue of its size (i.e. entrapment and encapsulation) will also effectively exclude the analyte from the majority, if not all, of the binding sites. Therefore, for such devices it is preferable, if not essential, to immobilise the antibody at the sensor/ sample interface.

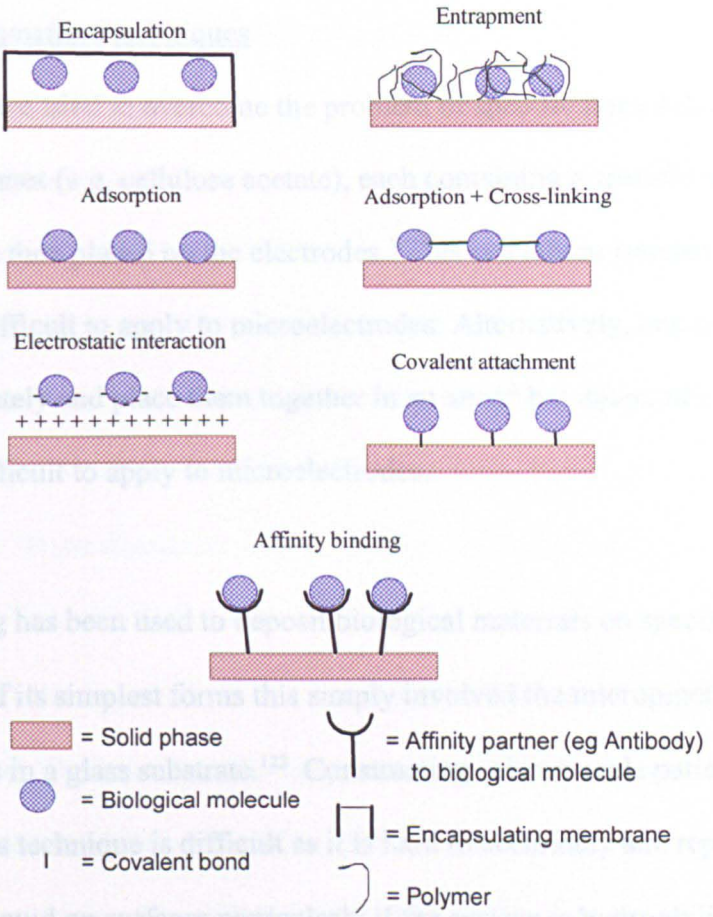

Figure 1.3 Classification of techniques for immobilisation of biomolecules. Encapsulation involves retaining molecules that are in solution or a gel, by the use of a semipermeable membrane. Entrapment involves the trapping of the biological molecule in a porous insoluble polymeric matrix. In adsorption the biological molecule sticks to the surface by a number of physical processes such as hydrophobic interactions with non-polar surfaces, this can be further stabilised by covalent cross-linking of the adsorbed molecules. Immobilisation can also by achieved by electrostatic interactions between the charge on the biological molecule and a charged surface. In covalent attachment the molecule is tethered to the surface via covalent chemical bonds, whilst affinity binding makes use of specific interactions between biological molecules to attach the desired molecule to the surface.

1.9 Review of possible immobilisation strategies

1.9.1 Physical deposition techniques

Some workers have tried to overcome the problem of specific immobilisation by casting different membranes (e.g. cellulose acetate), each containing a specific antibody. These membranes were then placed on the electrodes.[1] This is a labour intensive technique, and would be very difficult to apply to microelectrodes. Alternatively, one can construct the electrodes separately and place them together in an array[2] but again, this technique is labour intensive and difficult to apply to microelectrodes.

Micropositioning has been used to deposit biological materials on specific areas of a surface. In one of its simplest forms this simply involved the micropipetting of the species into etched wells in a glass substrate.[122] Constructing micron-scale patterns on a planar surface using this technique is difficult as it is hard to accurately and reproducibly control the spread of a liquid on surfaces particularly if the surface is hydrophilic. Other micropositioning methods include screen-printing and ink-jet printing techniques.

Screen-printing has been widely used for the selective deposition of biological molecules in the fabrication of biosensors.[133-136] Site specific immobilisation is achieved using screen printing by placing a stencil on top of the surface to be patterned. An ink which contains the species to be immobilised is coated over the stencil, and passes through holes onto the surface below. The resolution of the technique is limited to approximately 50 μm by a number of factors, particularly the nature of the surface and the rheology of the ink. This technique is generally used for immobilisation of proteins in relatively large patterns, but is not suitable for micron-scale patterning. The molecules tend not to be deposited as a

monolayer, which means that a considerable proportion may be inaccessible to the analyte, particularly in the case of antibody/antigen reactions.

Ink-jet printing technology has been used to specifically deposit biological molecules (principally enzymes) on surfaces.[137-139] In this technique an inlet from a reservoir allows an enzyme containing solution access into a pressure chamber, which is surrounded by a piezoelectric element. The application of an electrical pulse to the piezoelectric element induces contraction of the pressure chamber, thus forcing the solution through a small nozzle (typically 50 μm diameter) onto the surface. A 50 μm diameter hole results in a surface patterned area with a diameter of between 150 and 200 μm, and the distribution of the enzyme within this area tends to be uneven (typically much greater at the periphery of the patterned area). The use of smaller nozzles coupled with treatment of surfaces so that they are hydrophobic and thus resistant to spreading of solutions, has enabled an improvement by an order of magnitude for the minimum area to be patterned.[139] Air-jet printing is a similar technique, where an aerosol produced by mixing a solution stream with an air-jet and passing though a nozzle, is directed at the sensor surface.[140]

1.9.2 Photolithographic "lift-off" techniques

Molecular patterning has been achieved on planar surface using photolithographic processes.[141,142] This can be achieved in two ways as shown in Figure 1.4. In the first technique, the surface is coated with photoresist, which is patterned and developed. The surface is then coated with a hydrophobic silane, and "lift-off" is performed leaving a hydrophobic pattern on the surface. The remainder of the surface is coated with a hydrophilic silane to which proteins may subsequently be attached.

In the second technique, the surface is coated with aminosilane and then photoresist which is patterned and developed. A protein species is added and "lift-off" is performed leaving the patterned protein. A second protein species can be added to complete a 2 protein pattern. Details of creating photoresist patterns and the use of lift off techniques are given in Chapter 3. There are two major problems with this technology: firstly that a maximum of two proteins can be immobilised; and secondly that of these, only one is likely to be functional as the first patterned is exposed to organic solvent during lift-off, which can cause denaturation of the protein.[143,144]

A modified lift-off technique has been described for the patterning of enzymes in a multianalyte biosensor.[145] An enzyme solution containing glutaraldehyde was spin-coated onto a wafer covered with patterned photoresist. The resultant immobilised enzyme layer was removed by ultrasonic vibration in acetone, except in areas where there was no resist. Photoresist was again coated over the entire surface and repatterned. A second enzyme solution containing glutaraldehyde was spin coated onto the wafer, and again lift-off was used. A disadvantage of this process was that most of the enzyme solution was consumed without contributing to membrane fabrication, therefore the application was limited to use in sensors which utilise relatively inexpensive enzymes.[137]

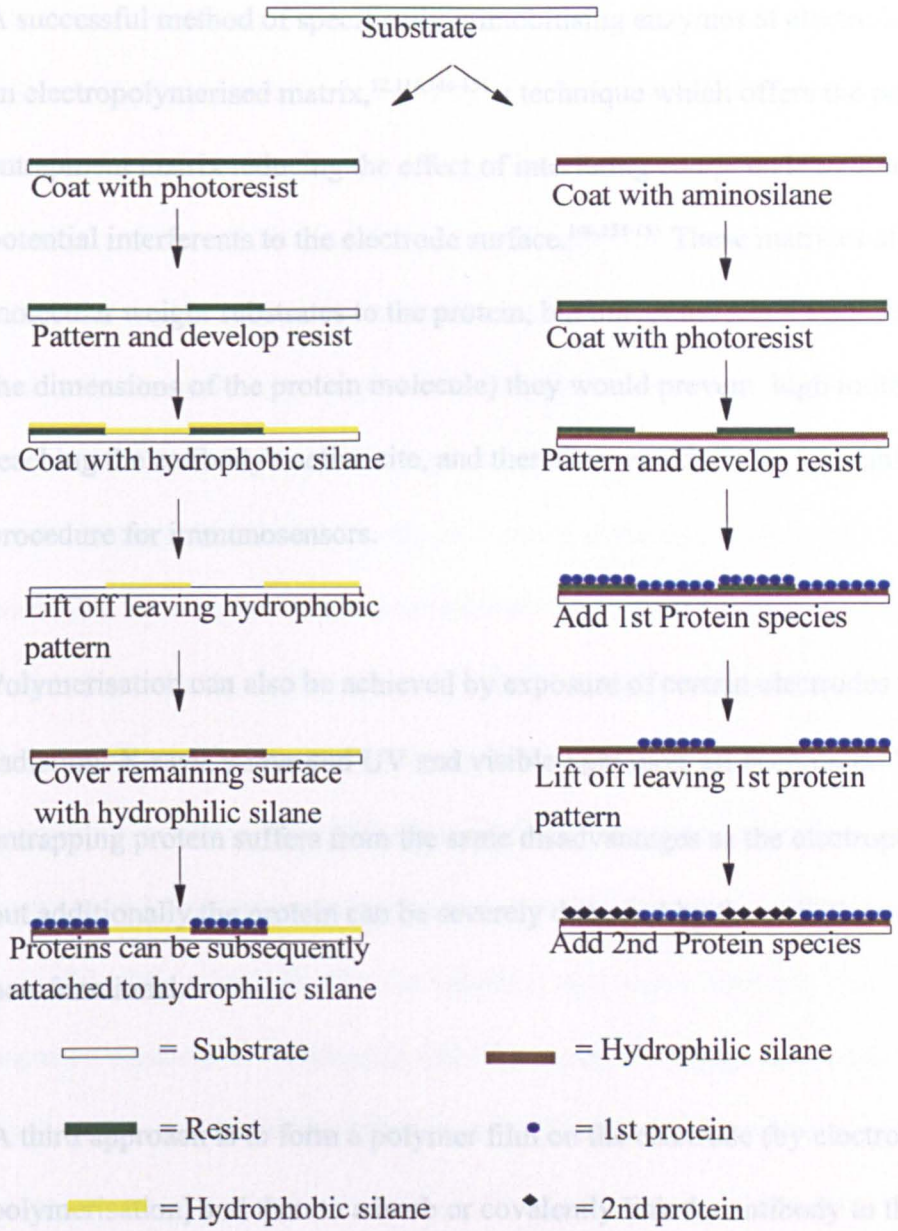

Figure 1.4 Techniques for molecular patterning using photoresist lift-off. In the first technique, the surface is coated with photoresist, which is patterned and developed. The surface is then coated with a hydrophobic silane, and "lift-off" is performed leaving a hydrophobic pattern on the surface. The remainder of the surface is coated with a hydrophilic silane to which proteins may subsequently be attached. In the second technique the surface is coated with aminosilane and then with photoresist which is patterned and developed. A protein species is added and "lift-off" is performed leaving the patterned protein. A second protein species can be added to complete a 2 protein pattern.

1.9.3 Entrapment in Polymers

A successful method of specifically immobilising enzymes at electrodes is to entrap them in an electropolymerised matrix,[12,116,146-155] a technique which offers the possibility of the entrapment matrix reducing the effect of interfering compounds by limiting access of potential interferents to the electrode surface.[146,153-155] These matrices allow access of low molecular weight substrates to the protein, but unless the films were very thin (thinner than the dimensions of the protein molecule) they would prevent high molecular weight antigen reaching the antibody's active site, and therefore would not be a suitable immobilisation procedure for immunosensors.

Polymerisation can also be achieved by exposure of certain electrodes to electromagnetic radiation. X-rays, γ-rays and UV and visible light have all been used.[156-158] This method of entrapping protein suffers from the same disadvantages as the electropolymerised matrix, but additionally the protein can be severely damaged by the radiation rendering it non-functional.

A third approach is to form a polymer film on the electrode (by electro- or photo-polymerisation) and then to adsorb or covalently link the antibody to the polymer.[159,160] Polymers have been used to influence the orientation of molecules and can provide direct electronic communication between a redox protein and an electrode.[161] A major problem with polymerisation techniques for patterning biological molecules for use in multianalyte immunosensors, is in preventing non-specific binding to the polymer or the uncoated electrodes.

1.9.4 Electrodeposition followed by covalent cross-linking.

The migration of proteins to electrically charged electrodes, followed by cross-linking with glutaraldehyde has been used, as a means of selectively depositing enzymes on electrode surfaces.[144,162] The migration of proteins in an electric field is dependent upon the isoelectric point (pI) of the protein and the pH of the supporting buffer. At pH values greater than the pI the protein molecule possesses a net negative charge, and will migrate towards a positively charged electrode. Once the protein has been deposited at the electrode, it can be secured by forming covalent linkages using glutaraldehyde as a crosslinker. The technique results in the formation of protein multilayers at the electrode, the thickness of which can be controlled by variation of the immobilisation conditions. It is generally considered that the protein layer that is in direct contact with the electrode surface is denatured and that protein functionality resides in ensuing layers.[163] A multianalyte sensor for the simultaneous determination of glucose and lactate has been fabricated using this technique, but it has not been applied to immunoassays due to a number of problems; firstly the technique is rather wasteful of the molecule to be immobilised as it is in a relatively thick layer, part of which has been denatured;[163] secondly with high molecular weight analytes, many of the antibody binding sites within the deposited layer will be unavailable to the antigen.

1.9.5 Techniques using deep ultraviolet light

The coating of the entire surface with protein followed by selective removal using deep ultraviolet (DUV) irradiation (λ = 193 nm) has been used to pattern IgG onto a surface.[143] The exact mechanism was not elucidated, but the irradiation caused either protein cleavage or denaturation in exposed areas. It was found, however, that the functionality of the remaining IgG was variable, and non-specific binding remained a major problem.

1.9.6 Langmuir-Blodgett

Orientation of immunoglobulins has been demonstrated using Langmuir-Blodgett (LB) films, thus maximising the immunoglobulin's efficacy.[164,165] Electrostatic interaction between proteins and Langmuir-Blodgett films has also been used as an immobilisation technique.[166] However, LB technology has not been successfully extended to patterning surfaces in a predefined manner, and the problems of specific immobilisation remain.

1.9.7 Self assembled monolayers

Patterns of hydrophilic and hydrophobic SAMs (each element 20μm in size) have been constructed on gold by using a stamp to attach an alkanethiol to selected areas of a gold surface (sulphur readily binds to gold),[167] a mercaptoalcohol is then attached to the remainder of the surface. SAMs have also been used to pattern a protein on a surface. In particular SAMs formed by the chemisorption of thiol alcohols, especially oligo(ethylene glycol)-terminated alkanethiols ($HS(CH_2)_m(OCH_2CH_2)_nOH$), have been shown to resist the adsorption of a variety of proteins.[168]

Patterns of pyruvate kinase, with resolution of a few μm, have been formed on gold by stamping the surface with $HS(CH_2)_{15}CH_3$ and then derivatising the rest of the surface with $HS(CH_2)_{11}(OCH_2CH_2)_6OH$, followed by protein adsorption as shown in Figure 1.5. A polydimethylsiloxane (PDMS) stamp was fabricated using a resist master pattern, this mask was peeled away and inked with an alkanethiol (hydrophobic) which was transferred as a pattern onto the gold surface by stamping. The surface was then exposed to a solution containing $HS(CH_2)_{11}(OCH_2CH_2)_6OH$ (hydrophilic), and upon subsequent exposure to protein, adsorption occurred at the hydrophobic but not the hydrophilic areas.

This technique has the potential of constructing very high resolution patterns, as resolution is not limited by the wavelength of light, however it is limited to the patterning of a single protein. Patterns of SAMs could be formed in a similar way using resist based lift-off techniques (lift-off described in Chapter 3). The use of electron beam lithography would allow patterns to be formed whose resolution is not limited by the wavelength of light.

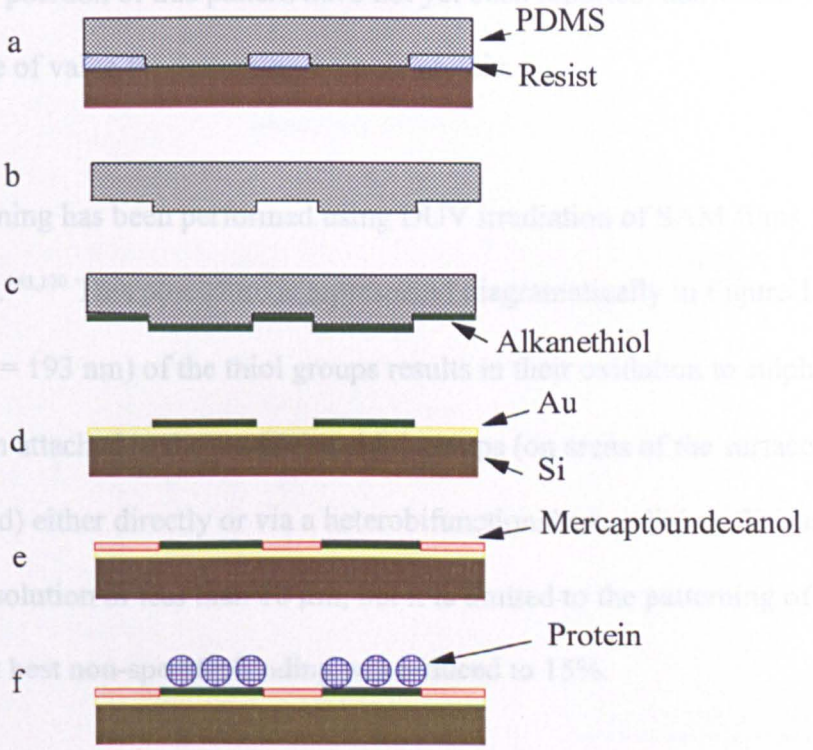

Figure 1.5. Schematic diagram for the construction of a protein pattern by means of stamping SAM films. (a) A polydimethylsiloxane (PDMS) stamp was fabricated using a resist master pattern, (b), this mask was peeled away and (c) inked with an alkanethiol (hydrophobic) which was (d) transferred as a pattern onto the gold surface by stamping. (e) The surface was then exposed to a solution containing $HS(CH_2)_{11}(OCH_2CH_2)_6OH$ (hydrophilic), and (f) upon subsequent exposure to protein, adsorption occurred at the hydrophobic but not the hydrophilic areas.

It has been suggested that self assembly combined with LB techniques could be used to pattern proteins on gold.[169] A mixture of palmitic acid and a sulphur containing lipid (thiolipid) was spread on the water/air interface of a Langmuir trough. On compression the components of the floating monolayer separated and domains were formed which

predominantly contained palmitic acid. The film was transferred to a gold substrate where the thiolipids covalently bound to the gold and the physisorbed palmitic acid was washed away to leave domains of bare gold to which other species such as proteins could be attached. This technique would have the advantage that resolution of patterning would not be limited by the wavelength of light as in photoactivation/deactivation or photolithographic techniques. However, methods for precisely controlling the size, geometry and position of this pattern have not yet been reported, additionally this technique would only be of value for patterning a single protein.

Protein patterning has been performed using DUV irradiation of SAM films of organosilanes.[143,170] This procedure is represented diagramatically in Figure 1.6. DUV irradiation (λ = 193 nm) of the thiol groups results in their oxidation to sulphonate groups. Protein is then attached to the remaining thiol groups (on areas of the surface that have not been irradiated) either directly or via a heterobifunctional cross-linker. This method achieved a resolution of less than 10 μm, but it is limited to the patterning of a single species and at best non-specific binding was reduced to 15%.

1.9.8 Masking using thioalkanes

It has been suggested that it is possible to mask electrodes to prevent antibody adsorption, whilst leaving other electrodes exposed so that the antibody can be immobilised, by the use of species (for example thioalkanes) that are adsorbed or desorbed at particular electrode potentials.[130] The successful application of this technique to patterning proteins on surfaces has not been reported. A potential disadvantage of these techniques is that the use of organic solvents is required which may affect the structural integrity and therefore the function of proteins.[144] Additionally, these techniques would be wasteful of biomolecules.

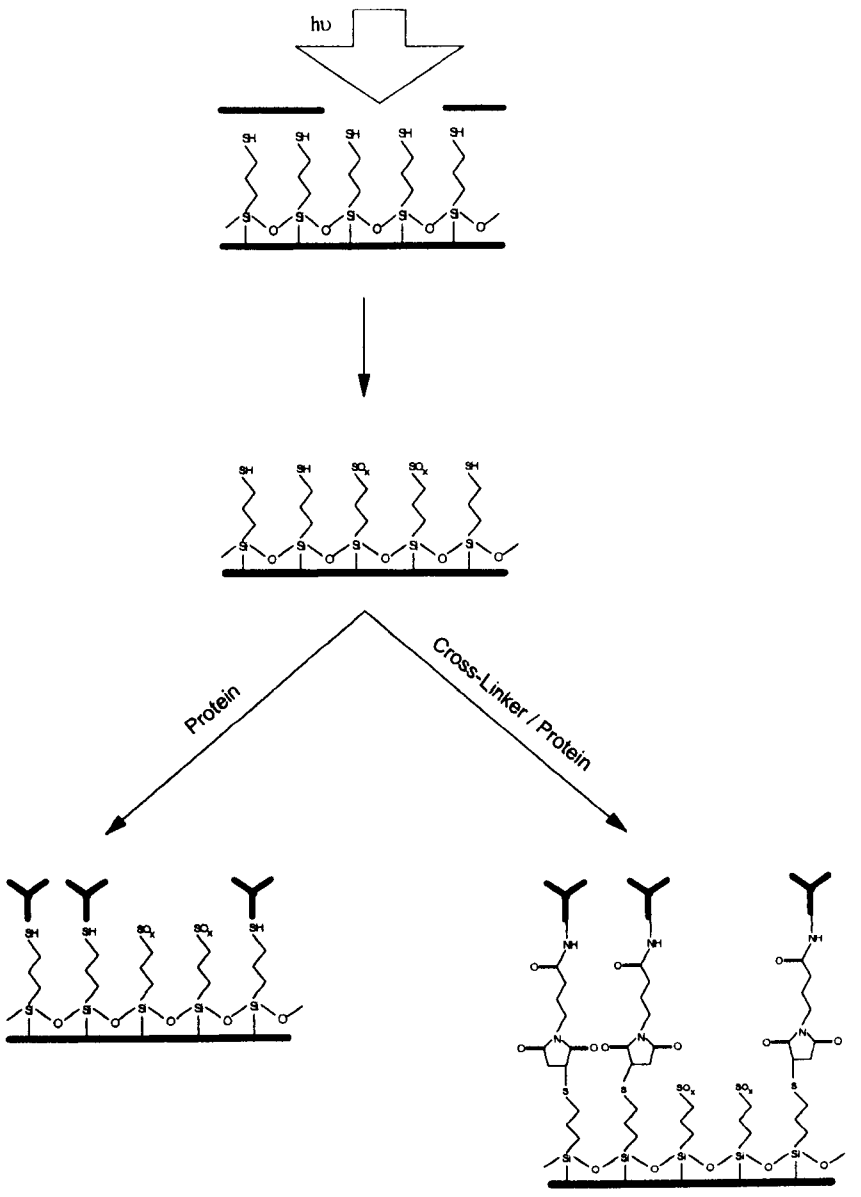

Figure 1.6 Schematic diagram for the patterning of proteins by DUV irradiation of SAM films. DUV irradiation (λ = 193 nm) of the thiol groups results in their oxidation to sulphonate groups. Protein is then attached to the remaining thiol groups (on areas of the surface that have not been irradiated) either directly or via a heterobifunctional cross-linker. Protein does not attach to the sulphonate groups.

1.9.9 Photoactivation and photodeactivation techniques

Fodor and co-workers have reported the light-directed spatially addressable parallel chemical synthesis of oligopeptides and oligonucleotides.[171,172] They have combined the use of solid phase chemistry, photolabile protecting groups and photolithography to synthesise a range of chemical products. The principle of the technique is illustrated in Figure 1.7. The substrate possesses amino groups that are blocked with a photolabile protecting group such as nitroveratryloxycarbonyl (Nvoc), illumination of specific regions through a lithographic mask results in photodeprotection. The exposed amino groups are thus available for coupling to another species (which has a photolabile protecting group attached) using conventional solid phase synthesis methods.[173] A different mask is used to photodeprotect different regions of the substrate, and a different photolabile protected group containing species is attached to the exposed amino groups. Additional cycles of photodeprotection and coupling are performed to obtain the desired set of products.

This technology is capable of rapidly synthesising large numbers of oligopeptides and oligonucleotides. With a simple binary mask, 256 different species can be synthesised in only 8 steps, the use of more complex masks can increase the number of species synthesised.[174] Resolution of between 10 and 20 μm has been achieved within the arrays of synthesised oligopeptides.[175]

The formation of a covalent linkage between the protein and the substrate by means of a photoactivatable link has previously been reported.[176] In some of these techniques, a photoactivatable function provided by an aryl azide (e.g. 3-nitro-4-aminophenyl azide) was attached directly to a silica surface. The modified surface was covered with an enzyme containing solution, and designated areas were exposed to light by using a mask. Assay of

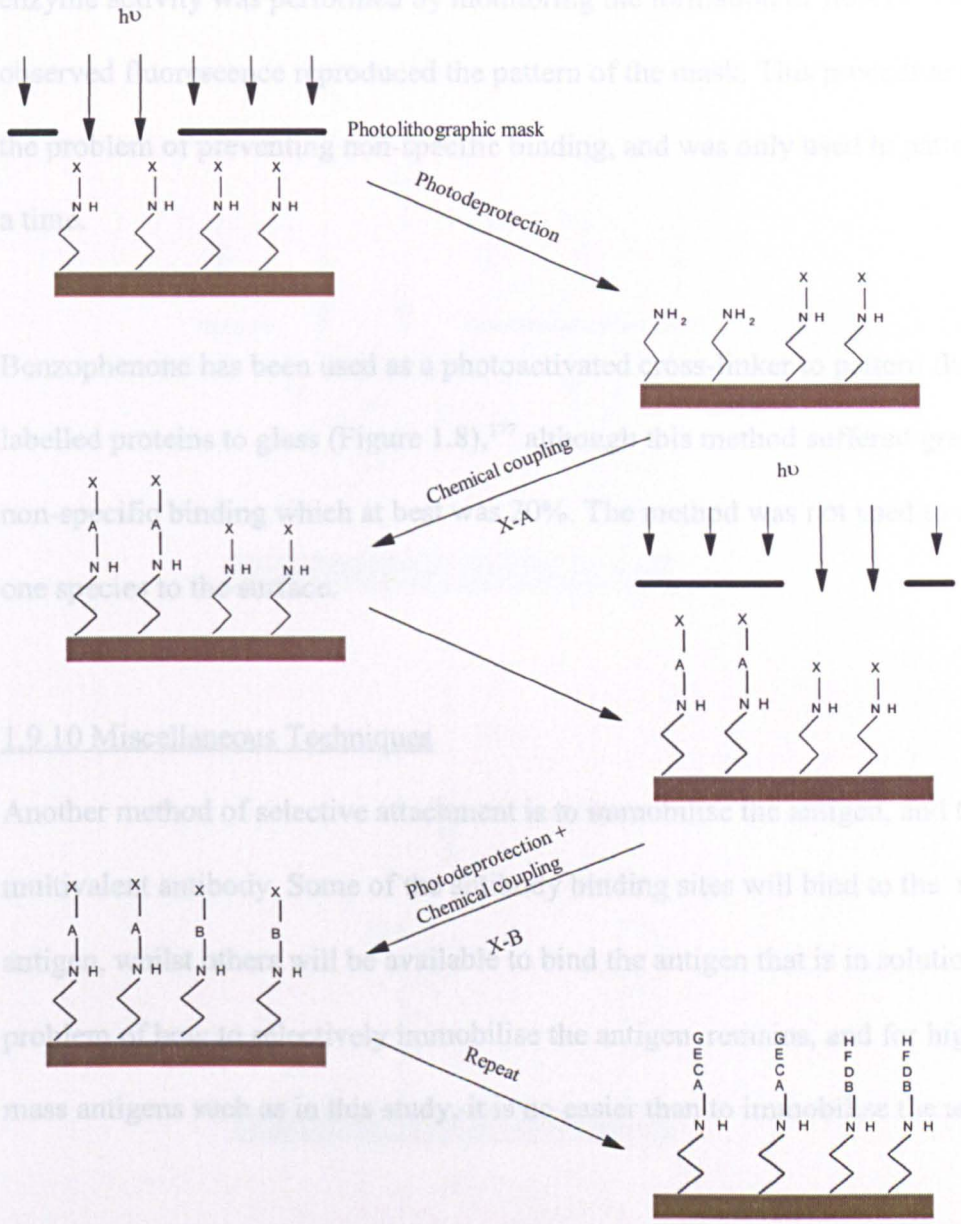

Figure 1.7 Schematic diagram for light-directed spatially addressable chemical synthesis. The substrate possesses amino groups that are blocked with a photolabile protecting group such as Nvoc. Illumination of specific regions through a lithographic mask results in photodeprotection. The exposed amino groups are thus available for coupling to another species; A (which has a photolabile protecting group (x) attached) using conventional solid phase synthesis methods. A different mask is used to photodeprotect different regions of the substrate, and a different photolabile protected group containing species is attached to the exposed amino groups. Additional cycles of photodeprotection and coupling are performed to obtain the desired set of products.

enzyme activity was performed by monitoring the formation of fluorescent products, the observed fluorescence reproduced the pattern of the mask. This procedure did not address the problem of preventing non-specific binding, and was only used to pattern one protein at a time.

Benzophenone has been used as a photoactivated cross-linker to pattern fluorescently labelled proteins to glass (Figure 1.8),[177] although this method suffered greatly from non-specific binding which at best was 20%. The method was not used to attach more than one species to the surface.

1.9.10 Miscellaneous Techniques

Another method of selective attachment is to immobilise the antigen, and then add a multivalent antibody. Some of the antibody binding sites will bind to the immobilised antigen, whilst others will be available to bind the antigen that is in solution.[178] The problem of how to selectively immobilise the antigen remains, and for high molecular mass antigens such as in this study, it is no easier than to immobilise the antibody itself.

Finally, functional antibodies and enzymes have been patterned on to surfaces by laser induced plasma deposition. In this technique biomolecules are vapourised and ionised by the laser, the ionised proteins are guided by electric fields towards a substrate on which they are deposited. Patterning can be achieved by placing a mask (copper grid) on the substrate, or by using spatially well defined electric fields. This technique has only been used to pattern one protein with a resolution of approximately 100 μm. The activity of the deposited protein over the surface tends to be non-uniform possibly due to deposition of large clusters consisting of several molecules.[179,180]

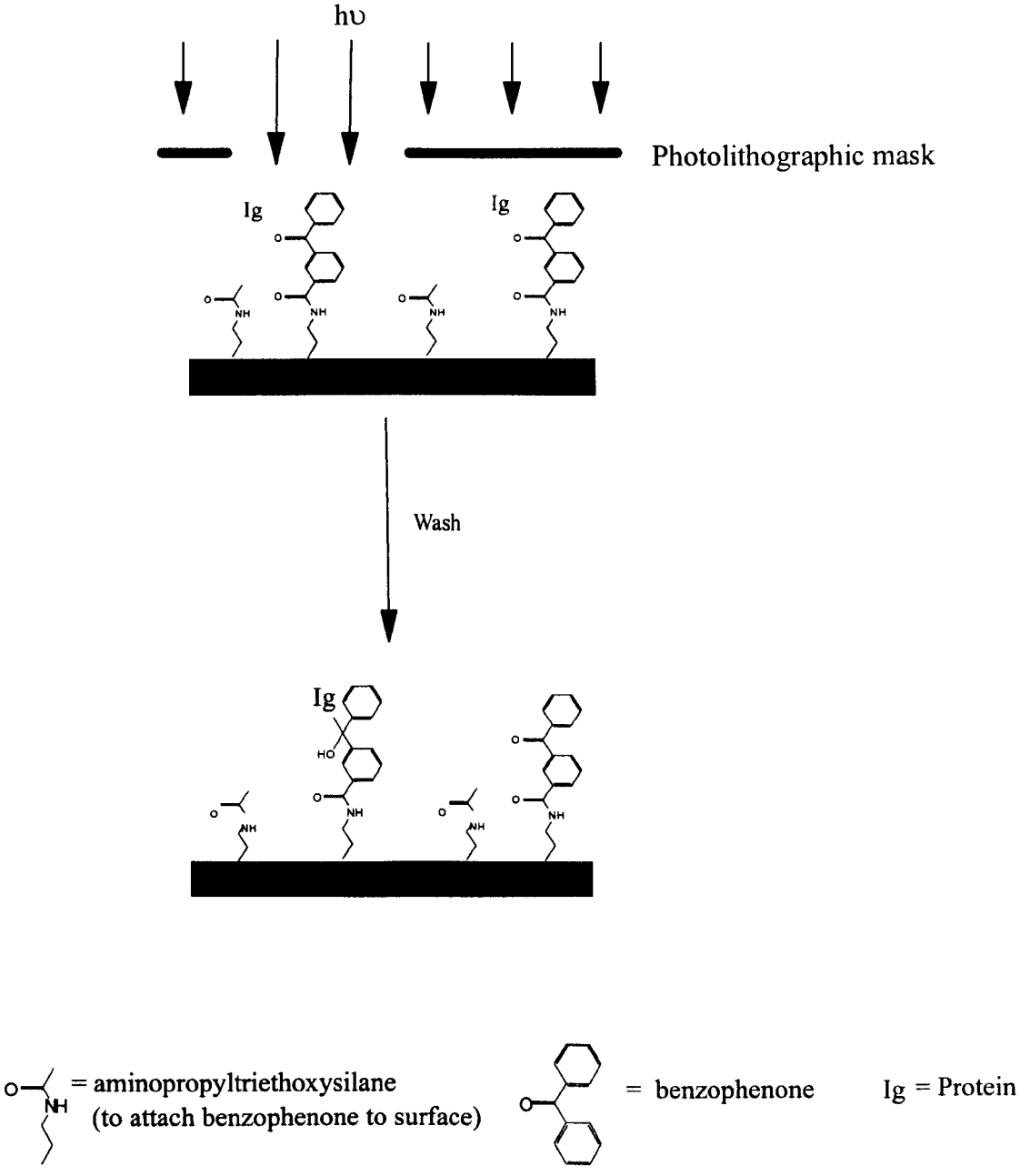

Figure 1.8 Use of benzophenone as a photoactivatable cross linker for protein immobilisation.
Benzophenone is attached to the surface via aminopropyltroethoxysilane (note that this is not 100% efficient). The benzophenone-derivatized surface is treated with a solution of the protein (Ig) and then irradiated through a lithographic mask. The protein molecules are bound covalently only in areas that are exposed to light.

CHAPTER 2

Se vogliamo che tutto rimanga come è,
bisogna che tutto cambi

(If we want things to stay where they are,
things will have to change)

Guiseppe di Lampedusa
Il Gattopardo (The Leopard)

2 Introduction to Initial Experimental Work on Protein Patterning Techniques.

Initial investigations were performed into the feasibility of using a variety of techniques for the site specific immobilisation of antibodies. Of the techniques studied, which included employing very thin polymer films and masking of electrodes using thioalkanes (data not shown), the use of a photoactivable binding agent appeared to be the most promising. Section 2.1 provides an introduction to the theory and principles, of the protein patterning methods, and of work carried out in investigating these techniques. Sections 2.2 to 2.12 provide materials used and detailed experimental methods. The remainder of the chapter (sections 2.13 to 2.22) presents the results obtained along with discussion of their significance.

2.1 The use of a Photoactivable Binding Agent for Immobilisation of Biological Molecules.

The possibility of patterning proteins by the use of photoactivable binding agents was investigated, particularly the use of a photoactive biotin analogue (photobiotin), in conjunction with avidin, a molecule with which biotin exhibits a specific affinity. Avidin is a protein with a molecular mass of approximately 60,000 found in egg whites (a microbial form streptavidin is obtained from Streptomyces avidinii) and possesses four binding sites for biotin (vitamin H) with which it forms a very stable complex, with an association constant $K_a = 10^{15}$ M^{-1} [181-183] corresponding to a free energy of association of about 330 KJ M^{-1} of tetramer (81 Kcal M^{-1}).[184,185] The role of avidin - biotin bonding in nature, and the significance of the bond strength is unknown, although avidin and biotin exhibit the highest known affinity between a protein and ligand, with a binding energy which is comparable to that of a covalent linkage.

Both avidin and streptavidin consist of 4 subunits, each of which is an 8 stranded antiparallel β sheet forming a β-barrel. The biotin binding site is situated near the end of the barrel and involves a highly stabilised network of polar and hydrophobic interactions.[181] Biotin can be conjugated to proteins or other biologically active species, and this complex can be attached to immobilised avidin.[186-189] The rationale in using the avidin - biotin system is based on the premise that if a biologically active species is modified with biotin through its side chain, the biological and physicochemical properties of the biotin modified molecule will not be significantly affected, and that the biotin is still available to bind to avidin or streptavidin. This system has been extensively used in immunoassay techniques.[189,190] Avidin tends to show a high degree of non-specific binding to substances which is usually attributed to two inherent characteristics of the native molecule; a high pI and the presence of carbohydrate residues. Streptavidin, which has a lower carbohydrate content, and deglycosylated avidin, both exhibit reduced non-specific binding as compared to intact avidin.[185,191] The high isoelectric point (pI 10.0 - 10.5) of avidin, as well as the presence of carbohydrate (which accounts for about 10% of avidin's total molecular weight) on the protein contribute to non-specific binding in applications involving avidin. Due to its more favourable isoelectric point and lack of carbohydrate, streptavidin has historically been the accepted substitute for applications where the non-specific binding characteristics of avidin can not be tolerated. More recently, modified forms of avidin have become available, with pI values closer to 7.0 and reduced carbohydrate content leading to improved non-specific binding characteristics.[192,193] Streptavidin and deglycosylated avidin have been utilised as coatings on immunoassay microtitre plates to minimise non-specific binding.[194]

Photobiotin is a photoactivable biotin analogue (N-(4-azido-2-nitrophenyl)-N'-(N-d-biotinyl-3-aminopropyl)-N'-methyl-1,3-propanediamine), which was developed as a nucleic acid hybridisation probe,[195] and has also been used to label proteins.[196] Photobiotin contains an aryl azide group which is stable in the dark, but upon exposure to ultraviolet / blue light (340 - 375 nm) a highly reactive aryl nitrene group is generated which will react with proteins (and other species).[196] The potential effects of steric hindrance preventing binding between an activated aryl nitrene group and a protein can be minimised by introducing a spacer arm into the photobiotin molecule.[195] Such a spacer arm contains basic tertiary amino groups, which are positively charged at neutral pH and give a high solubility in water. The structure of the long arm photobiotin used in this work is shown in Figure 2.1

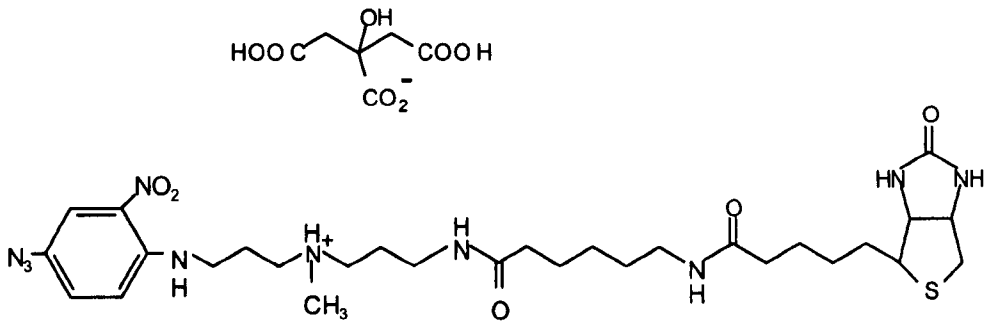

Figure 2.1 Chemical structure of long arm photobiotin. Photobiotin is a photoactivable biotin analogue, containing an aryl azide group which is stable in the dark, but upon exposure to ultraviolet / blue light (340 - 375 nm) a highly reactive aryl nitrene group is generated which will bind non-specifically to organic molecules. The introduction of a spacer arm minimises steric hindrance, and this spacer arm contains basic tertiary amino groups, which are positively charged at neutral pH and give a high solubility in water.

A number of approaches to the use of photobiotin and avidin in the immobilisation of molecules were examined;

a) Depositing a protein layer on the surface, adding photobiotin in the presence of light, followed by the attempted immobilisation of a second protein through avidin-biotin interactions.

b) Depositing a protein layer on the surface, adding avidinated protein with photobiotin attached and exposing to light;

c) Depositing an avidin layer on the surface, adding photobiotin followed by the addition of the species to be immobilised in the presence of light.

2.1.1 Depositing a protein layer on the surface, adding photobiotin followed by the immobilisation of a protein through avidin-biotin interactions

In this technique bovine serum albumin (BSA) was deposited on a surface which was subsequently exposed to light in the presence of photobiotin. This was followed by the sequential addition of avidin and a biotinylated protein, the rationale being that the avidin would bind to the immobilised biotin moiety and to the biotinylated protein resulting in the biotinylated protein's immobilisation. This is represented diagramatically in Figure 2.2. Initially, BSA was immobilised on to the surface (Figure 2.2a), photobiotin was added and exposure of selected areas of the surface to light through a mask resulted in activation of the photobiotin molecule (Figure 2.2b). Unbound photobiotin was removed by washing, and avidin was added which bound to the immobilised photobiotin groups (Figure 2.2c). A biotinylated protein was then added which bound to the immobilised avidin (Figure 2.2d).

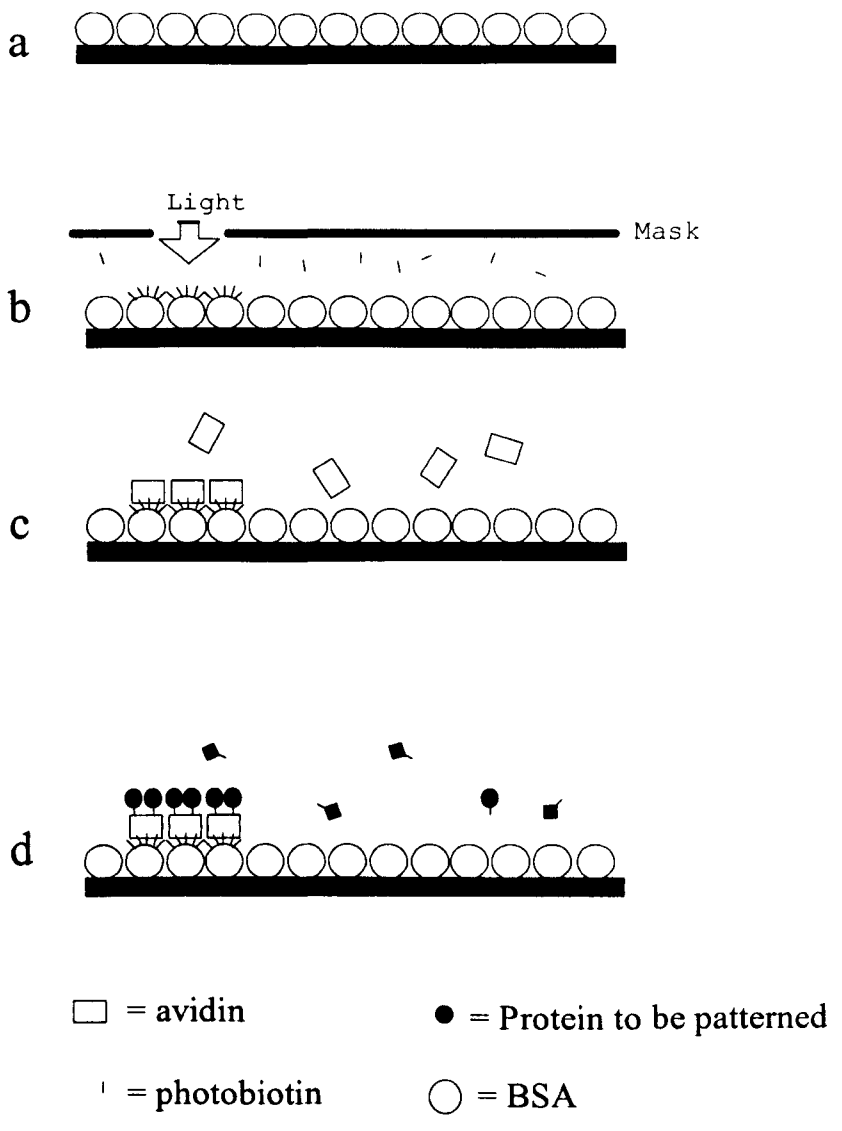

Figure 2.2 Scheme for depositing a protein layer on the surface adding photobiotin and immobilising proteins through avidin-biotin interactions. BSA was immobilised on to the surface (a), photobiotin was added and exposure of selected areas of the surface to light through a mask resulted in activation of the photobiotin molecule (b). Unbound photobiotin was removed by washing, and the surface was incubated with a solution of avidin which bound to the immobilised photobiotin groups (c). A biotinylated protein was then added which bound to the immobilised avidin (d).

2.1.2 Depositing a protein layer on the surface, and add avidinated protein with photobiotin attached

In this technique BSA was deposited on a surface, followed by the addition, in the presence of light, of an avidinated protein which had been preincubated with photobiotin. The rationale behind this method was that the photobiotin (and as a consequence of the avidin biotin interaction, the avidinated species), would bind to the protein coated surface. This is depicted diagramatically in Figure 2.3. Initially a protein - avidin - photobiotin complex was formed (Figure 2.3a), which was then placed in contact with a surface on which BSA had been immobilised (Figure 2.3b). Exposure of selected areas of the surface to light through a mask resulted in activation of the photobiotin molecule which attached to the surface BSA, thus immobilising the attached protein (Figure 2.3c).

2.1.3. Depositing an avidin layer on the surface, adding photobiotin followed by the addition of the species to be immobilised.

A third approach to protein patterning developed in this study was the use of photoactivable binding using photobiotin, which was bound to an avidin covered surface,[197-199] the immobilisation procedure is shown in Figure 2.4. Initially, avidin was immobilised on the surface and photobiotin was added and bound to the avidin (Figure 2.4a). Exposure of selected areas to light through a mask resulted in activation of the photobiotin molecule and a protein in the solution was immobilised specifically (Figure 2.4b). Unbound material was removed by washing, and the procedure repeated with a second protein (Figure 2.4c), the entire surface was then exposed to light in the presence of an agent such as casein in order to block previously unreacted photobiotin (Figure 2.4d).

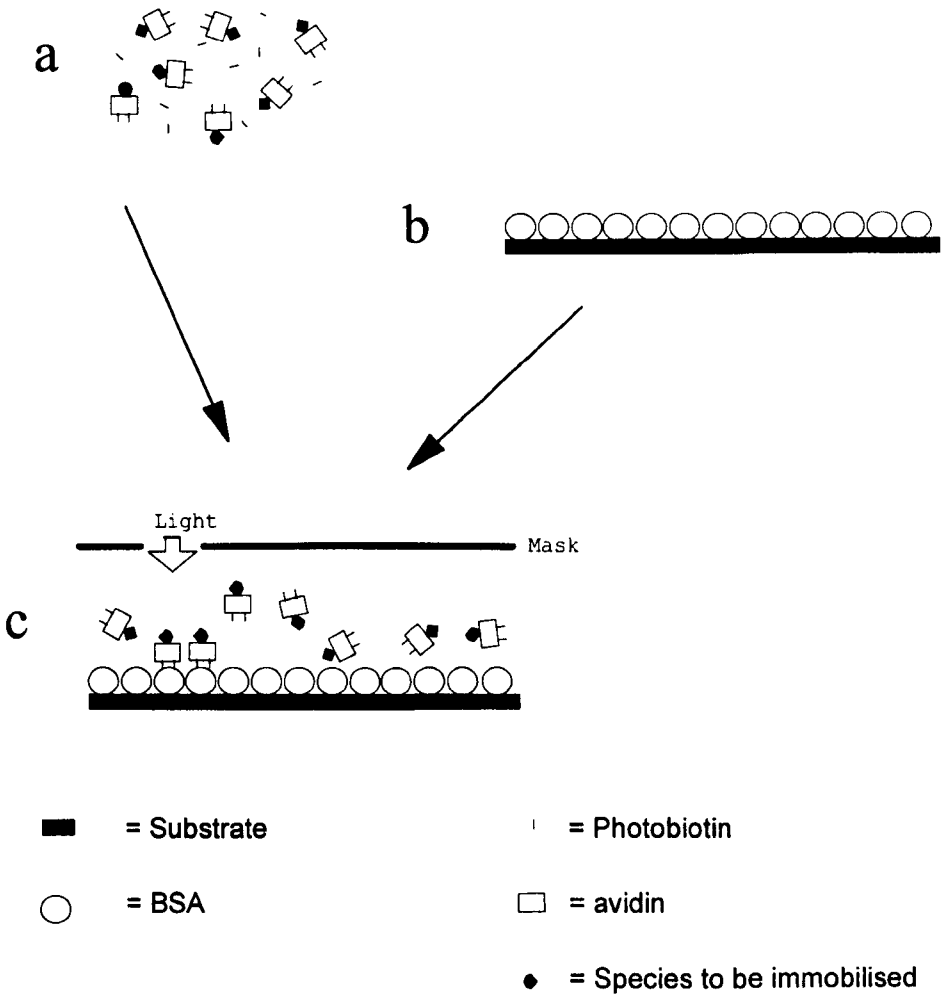

Figure 2.3 Scheme for immobilising an avidinated protein by depositing a protein layer on the surface and adding the avidinated protein with photobiotin attached. A protein - avidin - photobiotin complex was formed (a), which was then placed in contact with a surface on which BSA had been immobilised (b). Exposure of selected areas of the surface to light through a mask resulted in activation of the photobiotin molecule which attached to the surface BSA, thus immobilising the attached protein (c).

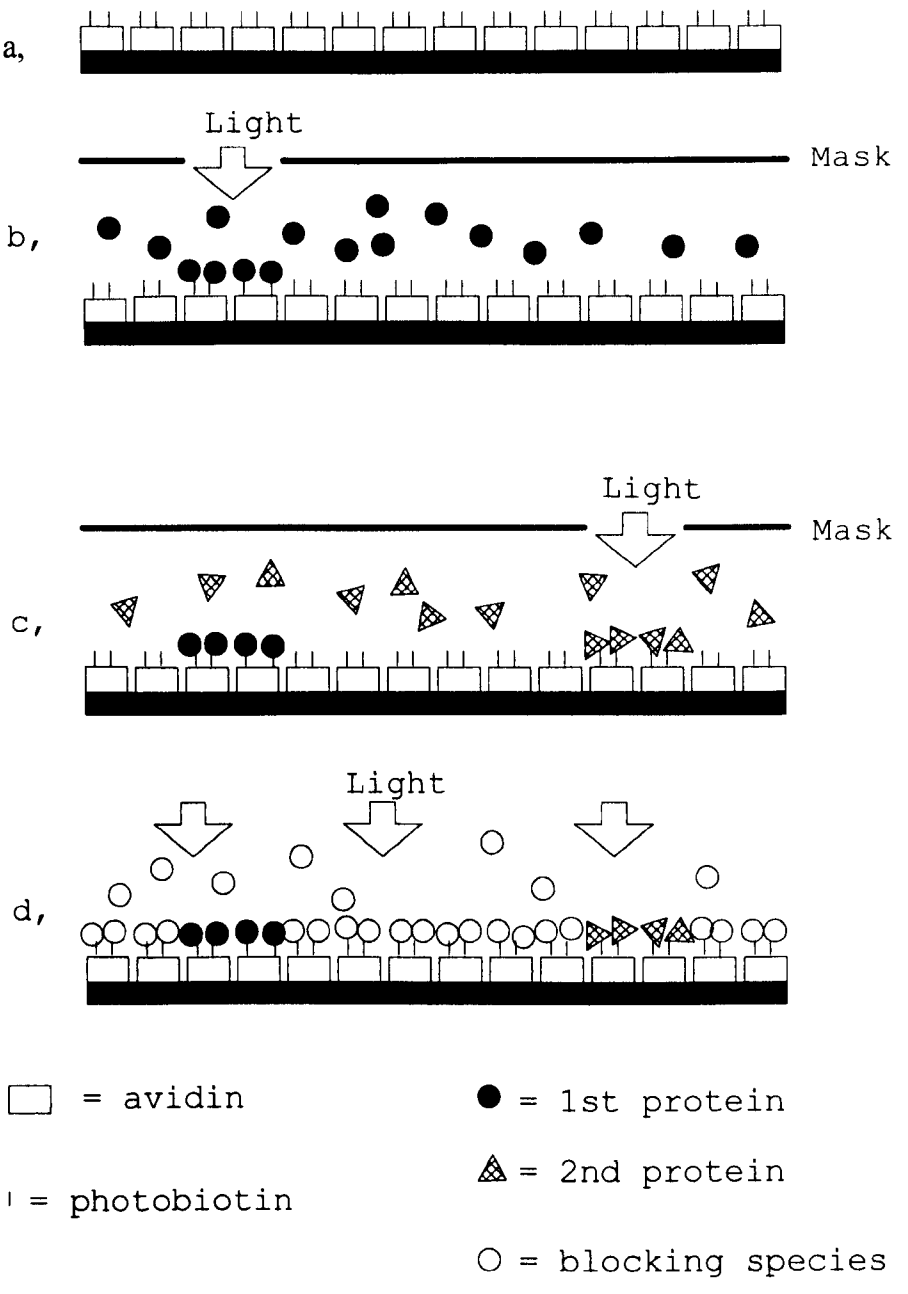

Figure 2.4. Scheme for immobilisation procedure coating the surface with avidin and photobiotin, followed by the addition of the protein to be patterned in the presence of light. Initially, avidin was immobilised on to the surface and photobiotin was added and bound to the avidin (a). Exposure of selected areas to light through a mask resulted in activation of the photobiotin molecule and a protein in the solution was immobilised specifically (b). Unbound material was removed by washing, and the procedure repeated with a second protein (c), the entire surface was then exposed to light in the presence of an agent such as casein in order to block previously unreacted photobiotin (d).

2.1.4 Investigation into resolution of avidin-photobiotin immobilisation procedure.

Investigations were performed to determine the resolution of patterned proteins that were achievable using the patterning technique that was selected as being the best of the methods (the criteria being relative efficiency of the immobilisation process, and contrast between patterned and non-patterned areas, results shown later in this chapter). This was done by patterning of proteins using a photolithographic mask with equal mark:space ratio lines of different widths. The patterned proteins were visualised by the addition of fluorescently labelled antibodies, and resolution was examined by measurement of the relative fluorescent intensity across the pattern.

2.1.5 Investigation into timescale of avidin-photobiotin immobilisation procedure.

The aim of this work was to determine the time required to complete the patterning process, under defined conditions (photobiotin being activated by light from a a 100W HB-10101AF super high pressure mercury vapour lamp (Nikon, Tokyo, Japan) situated 185 mm from the substrate (irradiance ~ 9 mW cm^{-2})). The distance of 185 mm was chosen, as this was the height of a chamber that was constructed to house samples being irradiated and providing protection to the user. Irradiance was directly measured by the use of a calibrated photovoltaic cell.

2.1.5.1 Activation of Photobiotin

The photoactivation of the photobiotin molecule (Figure 2.1) is due to the incorporation of an aryl azide. Aryl azides are derivatives of benzene and fused ring analogues, in which the azide (N_3) function is attached to a carbon that is part of the aromatic ring, i.e. all aryl

azides contain the group shown in Figure 2.5. The bonds in aryl azides are in equilibrium, as shown in Figure 2.6.

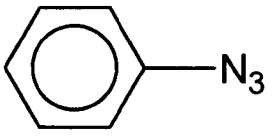

Figure 2.5 Structure of azidobenzene, the structure common to all aryl azides

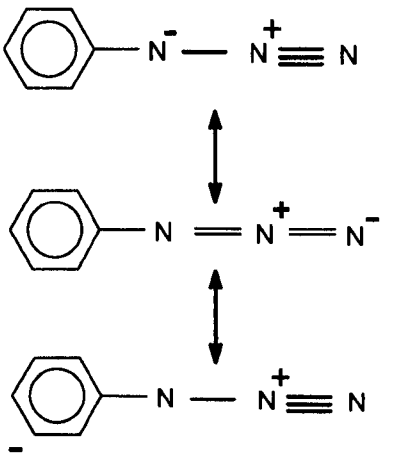

Figure 2.6 Equilibrium between bonds in aryl azide.

Aryl nitrenes are generated by thermolysis or photolysis of aryl azides which readily lose 2 nitrogen atoms as N_2 as shown in Equation 2.1

$$ArN_3 \xrightarrow[h]{\Delta \text{ or}} ArN + N_2 \quad (2.1)$$

Aryl nitrenes are extremely reactive and will undergo a large variety of reactions, some of which are shown in Figure 2.7.[200] Aryl nitrenes are very short lived species (half lives less than a second) but they may rearrange to give less reactive species, with longer half lives. Azidobenzene itself absorbs short - wavelength light (λ_{max} = 250 nm ε = 10,000) having a spectra with characteristic shoulders at 277 and 286-288 nm, but in aryl azides irradiation of the aromatic system allows transfer of energy to the azide function.[201,202] Nitro substitution of the aromatic ring leads to the appearance of an absorbance band at longer wavelengths so that light with a λ of around 350 nm will cause the production of nitrenes from the nitro substituted aryl azide present in photobiotin.

As the aryl nitrene group will bind to any organic molecules, the attachment of photobiotin to molecules should not be performed in buffers that contain organic material such as Tris(hydroxymethyl)methylamine (Tris).[203] Additionally, azides are rapidly converted to amines by thiol groups, commonly used in biochemical buffers in order to produce a reducing environment. The presence of electron withdrawing groups on the aromatic ring may be used to increase the reactivity of aryl nitrenes. The inclusion of such groups also affects the specificity of the reaction, making reactions with nucleophiles much more rapid at the expense of insertion reactions.[204]

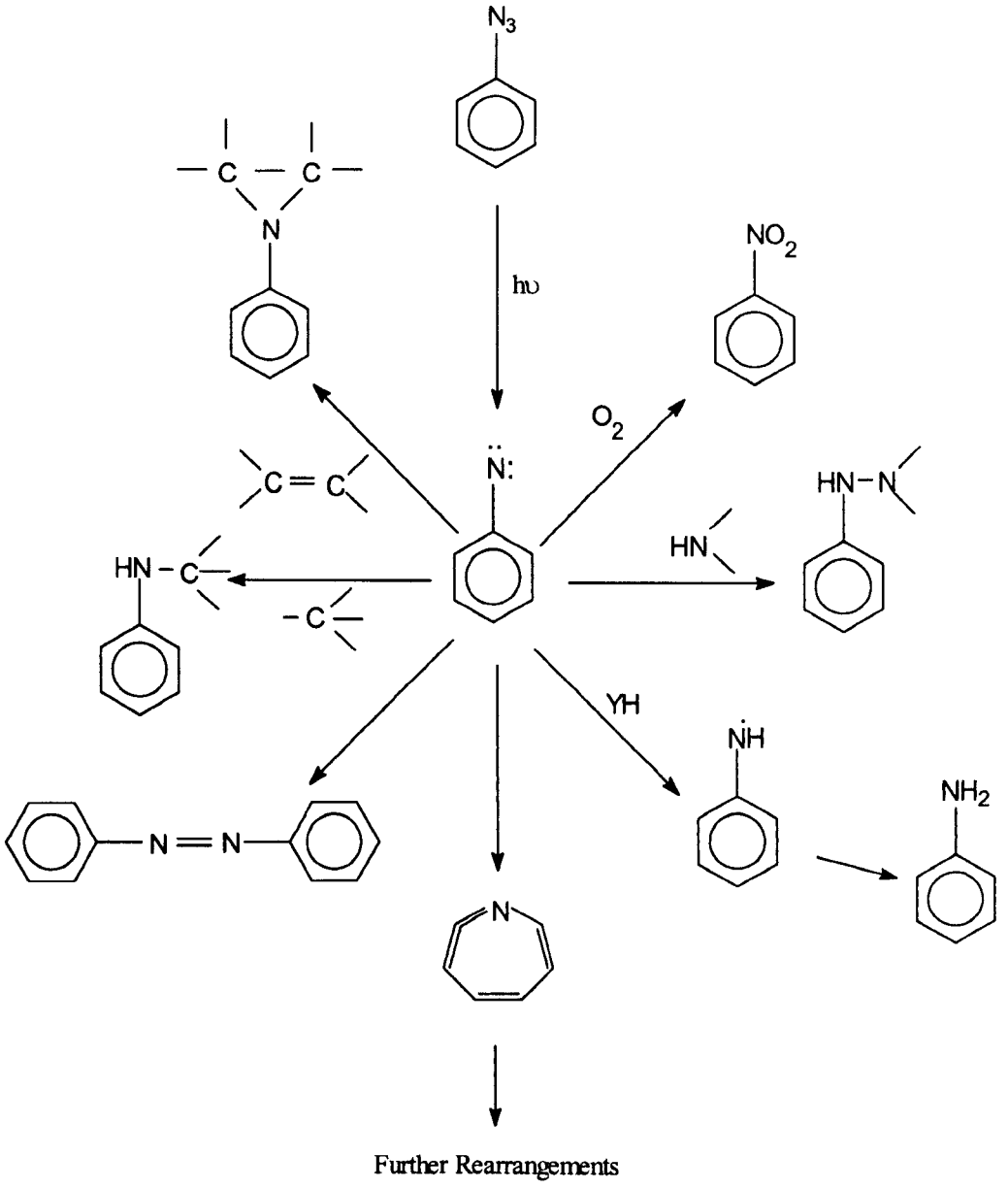

Figure 2.7 Some of the reaction of aryl azides upon activation.[200] They can react with amine groups and saturated and unsaturated carbons. Alternatively they can react with oxygen or acids, dimerise or undergo spontaneous rearrangements. [200]

2.1.6 Light activated patterning of multiple proteins on a silicon dioxide surface.

Once a patterning method had been selected (criteria being relative efficiency of the immobilisation process, and contrast between patterned and non-patterned areas, results shown later in this chapter), further work was performed to ascertain the possibility of patterning multiple proteins using this technique. Initially this was using just two proteins, but further work was performed to demonstrate the application of the developed patterning technique to the site-specific attachment of six different proteins (including 5 functional antibodies) within an area of 200 × 50 μm.

2.1.7 Demonstration of light-dependent coupling of enzymes and antibodies to gold, glass and carbon surfaces

Once a patterning method for the immobilisation of proteins on silicon dioxide had been selected, further work was performed to demonstrate the application of the immobilisation procedure to a range of surfaces, and to examine further characteristics of the technique. The aim of this section of work was to demonstrate the application of the patterning technique to the immobilisation of enzymes (GOD) and/or antibodies on to gold, glass and carbon surfaces. Successfully patterning enzymes and antibodies onto gold and/or carbon would allow the technique to be used for the fabrication of multianalyte amperometric enzymic biosensors and immunosensors.

2.1.8 Investigation into reducing non-specific binding on gold surfaces.

For the protein patterning to work efficiently, and in order to use the technique to develop a multianalyte electrochemical immunosensor, it was necessary to minimise non-specific binding of proteins to the surface. The effect upon non-specific binding of using different forms of avidin, different avidin immobilisation methods and different blocking reagents were examined. The types of avidin used were streptavidin (Vector) and two deglycosylated forms of avidin; avidin D (Vector) and Neutravidin (Pierce). The avidin immobilisation methods employed were adsorption, using N-acetyl-l-cysteine (NAC) with 1-ethyl-3-(3-dimethyl- aminopropyl)- carbodiimide (EDC), and using 3,3'-dithiobis(sulfosuccinimidylpropionate) (DTSSP). The three potential blocking agents examined were BSA, casein and Superblock™.

2.1.8.1 Immobilisation of avidin on gold using carbodiimide coupling to a N-acetyl-cysteine self assembled monolayer.

In this procedure NAC was assembled onto a gold surface through chemisorption of the thiol group. Subsequent attachment of the avidin to the NAC was by means of a carbodiimide. Carbodiimides comprise a group of compounds with the general formula R-N=C=N-R', where R and R' can be aliphatic or aromatic. This group of compounds has long been used for the synthesis of peptide bonds.[205] The carbodiimide used in this work was 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC).

The reaction of carbodiimides may be represented as a dehydration via an intermediary as shown in Figure 2.8. [205-207] (Temperatures of around 0°C favours the formation of peptide bonds, at higher temperatures the reaction shifts towards the formation of acyl ureas [207] (Figure 2.9)).

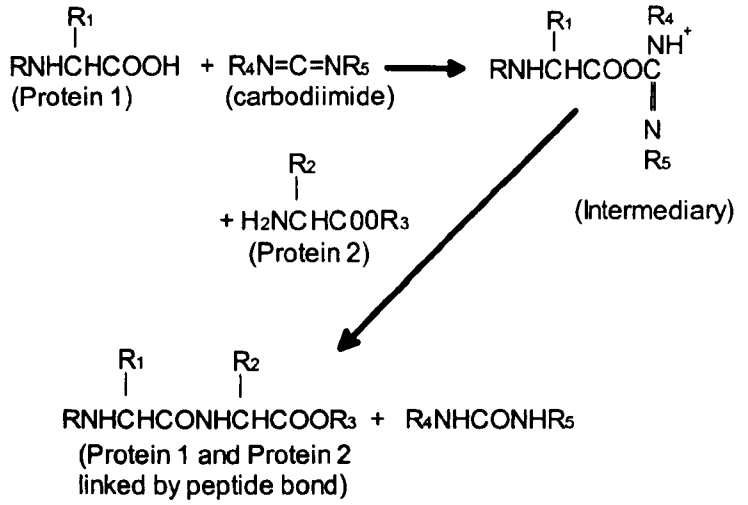

Figure 2.8 Reaction scheme for the promotion of peptide bond formation between amino and carboxylic acid residues in two proteins by use of carbodiimides.

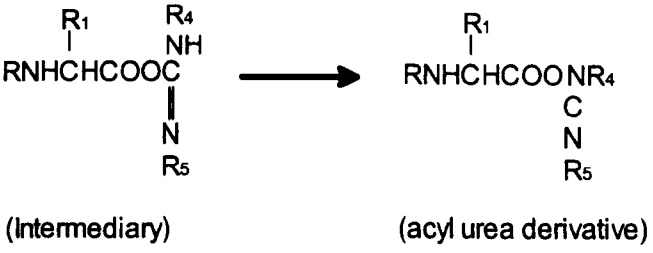

Figure 2.9 Reaction scheme for the formation of acyl ureas from carbodiimides attached to a carboxylic acid.

2.1.8.2 Immobilisation of avidin on gold using DTSSP

The structure of the 3,3'-dithiobis(sulfosuccinimidylpropionate) (DTSSP) is shown in Figure 2.10. DTSSP possesses two N-hydroxysuccinimide (NHS) esters connected by a disulphide linkage. The sulphur atoms in the disulphide bond will chemisorb to gold surfaces with a stability greater than that given by covalent silane bonds with glass. NHS esters react with primary amine - containing species to from stable amide (peptide) bonds (Figure 2.11). NHS esters also react with thiols.

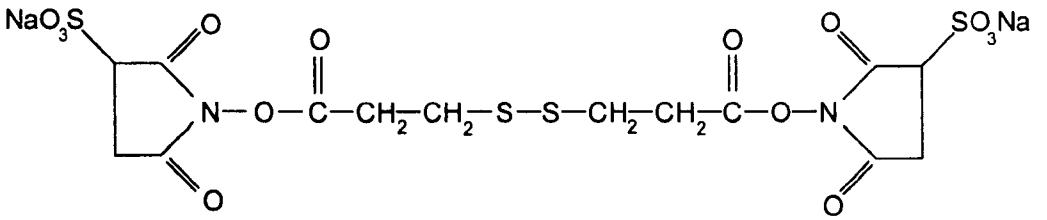

Figure 2.10 Structure of DTSSP (3,3'-dithiobis(sulfosuccinimidylpropionate)). The molecule possesses two N-hydroxysuccinimde (NHS) esters connected by a disulphide linkage.

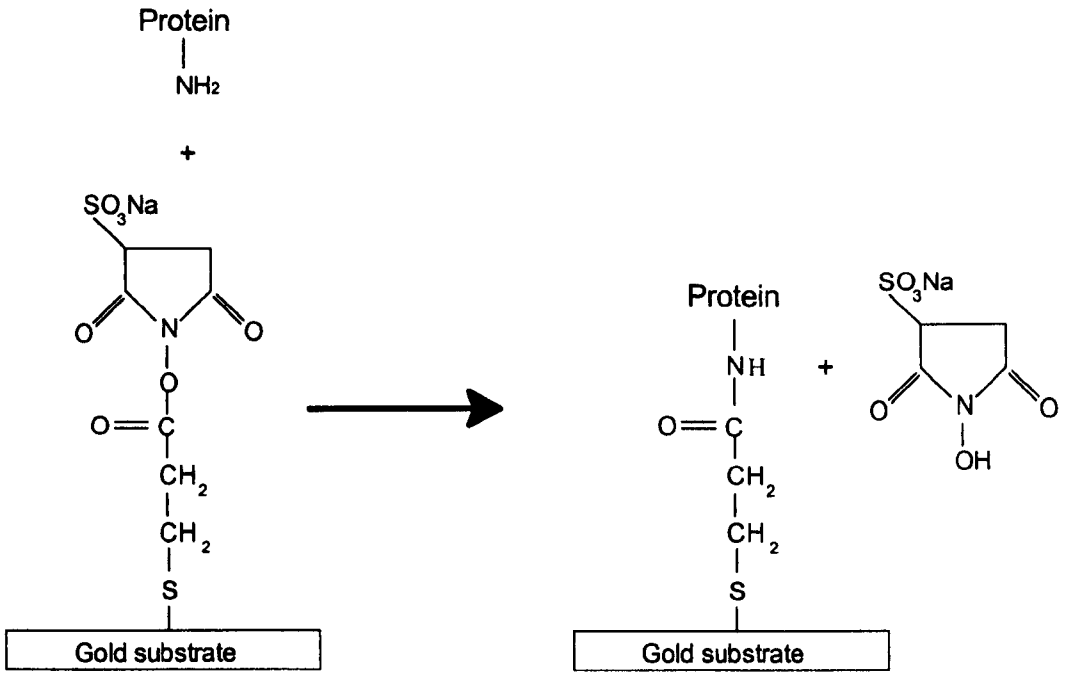

Figure 2.11 Coupling of protein to a DTSSP activated gold surface. The sulphur atoms in the disulphide bond chemisorb to gold surfaces with great stability. NHS esters react with primary amine - containing species (e.g. proteins) to from stable amide (peptide) bonds.

2.1.9 Regeneration of avidinated surfaces

Investigations were performed to elucidate if it was possible to break the avidin - photobiotin bond, whilst the avidin remained immobilised on the surface. pH extremes and chemicals (urea and guanidine) which are known to disrupt non-covalent bonds,[208-210] were examined. Being able to break the avidin-photobiotin bond, whilst leaving the immobilised avidin intact would introduce the possibility of regenerating and repatterning the surface, which could be advantageous if one wished to repattern and reuse the transducer element of an immunosensor (which is desirable if using expensive transducer elements).

2.1.10 Characterisation of biomolecular patterns using atomic force microscopy and fluorescence microscopy.

Atomic force microscopy was utilised to ascertain if information on the avidin-photobiotin patterning technique could be obtained, and comparing this information with data from fluorescent microscopy. Since its invention a little over a decade ago[211] scanning probe microscopy (SPM) has rapidly become a powerful technique for the imaging and analysis of surfaces. Scanning probe microscopy, (including atomic force microscopy (AFM) and scanning tunnelling microscopy (STM)) has been used to study the structure of immobilised biological molecules.[212-214]

The principle of scanning tunnelling microscopy (STM) is simple, when a bias voltage (0.1-1.0 V) is applied to a metallic tip, and advanced towards a conducting surface a tunnelling current is initiated at an appropriate distance from the surface (0.1-10 nm), due to electrons tunnelling from the tip through the gap to the surface. The size of the current is dependent upon on the work function of the tip and the bias voltage and is very sensitive to

the gap distance. Typically a change in distance of 0.1 nm results in an order of magnitude change in current. This critical relationship between distance and current gives rise to the vertical resolution of the technique.

Typically, in an experiment the tip is rastered across the surface controlled by a piezoscanner and the height of the tip above the surface is kept constant by means of feedback control (constant current mode), thus providing an image of the surface topography. Alternatively the tip is kept at a constant absolute height and changes in the current are recorded (constant height mode). STM requires that both the tip and the surface to be conducting. The advantage of STM over scanning electron microscopy (SEM) is primarily that of resolution, STM being capable of atomic resolution for flat surface,[215] and molecular resolution when imaging biomolecules.[214] Additionally, STM unlike SEM does not require the use of a high vacuum and images can be obtained at ambient temperature and pressure.

In AFM the elastic response of molecules to the force exerted by a scanning tip are measured. As the tip is rastered over the surface, changes in the surface cause small deflections in a spring mounted tip which can be measured and processed to give a 3 - dimensional image of the surface. The great advantage of AFM as compared with STM is that it can be used to image non-conducting surfaces. The main problem in imaging "soft" organic and biological material with an AFM is that the relatively high force exerted by the cantilever tip onto the sample may cause distortion or it may even be destructive.[216,217]

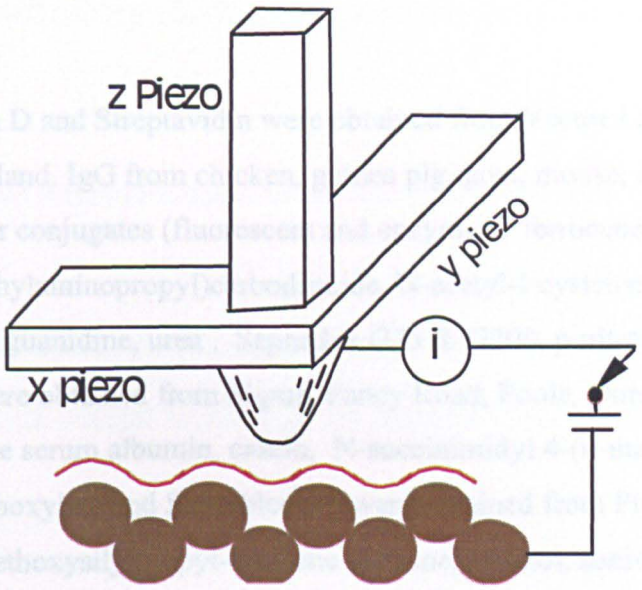

Figure 2.12. Diagram showing principle of operation of scanning tunnelling microscope. The tip is rastered across the surface controlled by a piezoscanner and the height of the tip above the surface is kept constant by means of feedback control (constant current mode), thus providing an image of the surface topography (represented by the red line). Alternatively the tip is kept at a constant absolute height and changes in the current are recorded (constant height mode).

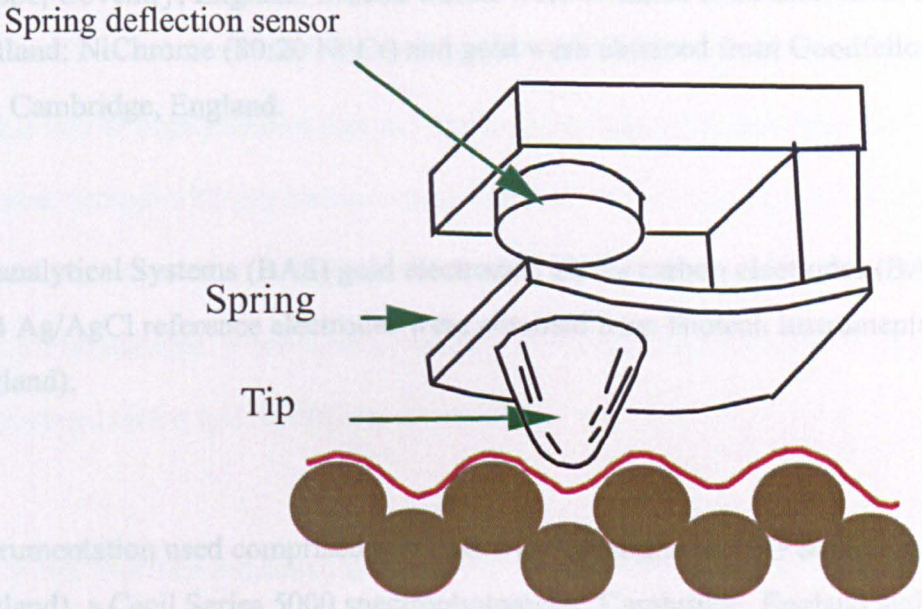

Figure 2.13. Diagram showing principle of operation of atomic force microscope. The tip is rastered over the surface, changes in the surface cause small deflections in the spring mounted tip (represented by the red line) which can be measured and processed to give a 3 - dimensional image of the surface.

2. 2 Materials

Photobiotin, avidin D and Streptavidin were obtained from Vector Laboratories, Peterborough, England. IgG from chicken, guinea pig, goat, mouse, rabbit and rat, all antibodies and their conjugates (fluorescent and enzymic), ferrocenemonocarboxylic acid, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, N-acetyl-l-cysteine, sodium cyanoborohydride, guanidine, urea , Sephadex G25 & G200, p-nitrophenol phosphate and glucose oxidase were obtained from Sigma, Fancy Road, Poole, Dorset, England. Neutravidin, bovine serum albumin, casein, N-succinimidyl 4-(n-maleimido- methyl)-cyclohexane-1-carboxylate and Superblock™ were obtained from Pierce Warriner, Chester, England. 1,3-trimethoxysilylpropyl-ethylene diamine, ethanol, sodium dihydrogen phosphate, disodium hydrogen phosphate, sodium chloride, glutaraldehyde, Tris, hydrochloric acid, dimethylformamide, zinc chloride and magnesium chloride were obtained from BDH, The Old Brickyard, Poole, Dorset, England.

Positive S1400-31 photoresist and Microposit developer were obtained from Shipley Europe, Coventry, England. Silicon wafers were obtained from Shin Etsu, Livingston, Scotland; NiChrome (80:20 Ni:Cr) and gold were obtained from Goodfellow Cambridge Ltd, Cambridge, England.

Bioanalytical Systems (BAS) gold electrodes, glassy carbon electrodes (BAS) and BAS RE4 Ag/AgCl reference electrodes were obtained from Biotech Instruments Ltd (Luton, England).

Instrumentation used comprised a EG&G 273A potentiostat (EG & G, Sunninghill, England), a Cecil Series 5000 spectrophotometer, Cambridge, England and a 100W HB-10101AF super high pressure mercury vapour lamp, Nikon, Tokyo, Japan.

2.3 Methods for Patterning of Proteins using Avidin and Photobiotin.

The use of photobiotin and avidin in the site specific immobilisation of molecules were examined using three different formats as outlined in sections 2.1.1 to 2.1.3.

2.3.1 Method for patterning of an avidin-protein conjugate by depositing a protein layer on the surface, adding photobiotin followed by the addition of the avidinated protein.

A SiO_2 wafer was immersed in 1% 1,3-trimethoxysilylpropyl-ethylene diamine in 95:5 (v/v) ethanol / distilled water for 120 seconds and briefly rinsed in 95:5 (v/v) ethanol / distilled water before heating at 120°C for 30 minutes. The wafer was immersed in 2% glutaraldehyde in 100 mM phosphate buffered saline pH 7.4 (PBS) for 15 minutes, and in 40mM sodium cyanoborohydride, 0.2mg ml^{-1} BSA in PBS for 30 minutes. The SiO_2 substrate was washed in PBS after this and all subsequent steps. The BSA-modified wafer was incubated in 5 ml of 10 $\mu g\ ml^{-1}$ long arm photobiotin in PBS whilst exposed to light from a 100 W high pressure mercury vapour lamp sited 185 mm from the wafer, for 15 minutes through a 10 μm interval equal mark space ratio mask (that had been fabricated photolithographically), following which the wafer was incubated in 20 $\mu g\ ml^{-1}$ avidin in PBS for 60 minutes. After washing in PBS, the wafer was incubated in 20 $\mu g\ ml^{-1}$ biotinylated rabbit IgG in PBS for 60 minutes.

The wafer was then incubated in 10 $\mu g\ ml^{-1}$ tetramethylrhodamine isothiocyanate (TRITC) labelled goat anti-rabbit IgG in PBS, for 60 minutes. After washing in PBS and distilled water the sample was dried in a stream of nitrogen and examined using fluorescence microscopy. Results are shown in Section 2.13.1, a photomicrograph of the resultant surface

is shown in Figure 2.14 and a cross sectional profile showing fluorescent intensity is shown in Figure 2.15.

Profiles of relative fluorescent intensity were obtained by digitising images by scanning into a computer using Photoshop™ software. A plot of relative fluorescent intensity was then produced by assigning a value of 100 to the brightest point (i.e. greatest fluorescence) on a line across the pattern. A fluorescent intensity of 0 was set as a point that would show no fluorescence (i.e. totally black). It should be noted that this zero value was based upon the software definition of black, and that it was not obtained from the photographic image. This was done so that a more objective measure of contrast and non-specific binding could be obtained.

2.3.1.1 Method for patterning avidin by depositing a protein layer on the surface, adding photobiotin followed by the addition of avidin

BSA was coated on to a SiO_2 surface, and photobiotin was subsequently attached as described in Section 2.3.1, following which the wafer was incubated in 20 μg ml^{-1} avidin-TRITC in PBS for 60 minutes. After washing in PBS and distilled water the sample was dried in a stream of nitrogen and examined using fluorescence microscopy. Results are shown in Section 2.13.1.

2.3.2 Method for patterning a protein by depositing a protein layer on the surface, and add avidinated protein with photobiotin attached

Avidin D was conjugated to rabbit IgG by using a maleimide as the conjugation reagent. 8 mg ml^{-1} avidin D was dissolved in 50 mM sodium borate buffer, pH 7.6 containing 1mM $MgCl_2$ and 0.1 mM $ZnCl_2$. To 1.0 ml of this avidin solution was added 1 mg of N-succinimidyl 4-(n-maleimido- methyl)cyclohexane-1-carboxylate (dissolved in 50 µl of N, N-dimethylformamide), and the mixture was incubated at room temperature for 1 hour. The reaction mixture was then applied to a Sephadex G-25 column (1.0 × 10 cm) equilibrated with 100 mM Tris-HCl buffer pH 7.0. Reaction products were eluted using the same buffer, and 1.0 ml fractions were collected. The protein content of these fractions was assessed by the measurement of absorbance at 280 nm using a Cecil Series 5000 spectrophotometer, and protein containing fractions were pooled and concentrated to 1.0 ml. Subsequently, 2.5 mg of rabbit IgG was dissolved in 0.5 ml of 50 mM sodium acetate buffer, pH 5.0 and this was added to the maleimide- avidin preparation and incubated at 4°C for 20 hours. 20 µl of 100 mM 2-mercaptotoethylamine was added to the reaction mixture and incubated at room temperature for 30 minutes to block remaining maleimide groups.

In order to separate the conjugated and unreacted material, the reaction mixture was applied to a Sephadex G-200 column (1.0 × 10 cm) equilibrated with 100 mM Tris-HCl buffer pH 7.0. Elution of reaction products was undertaken using the same buffer, and 1.0 ml fractions were collected. The relative protein content of these fractions was assessed by the measurement of absorbance at 280 nm and those that were earliest eluting (therefore having the highest molecular weight) were used as the avidin-IgG conjugate. The integrity of the conjugate was validated by use of a colourimetric ELISA, where the capture antibody was directed against avidin and the conjugate was a goat anti-rabbit IgG-alkaline phosphatase.

BSA was immobilised on a SiO_2 wafer using the silanisation procedure described in Section 2.3.1. 2.5 ml of 20 μg ml^{-1} avidinated rabbit IgG was incubated with 2.5 ml of 20 μg ml^{-1} long arm photobiotin in PBS for 20 minutes. This solution was then placed on the protein coated SiO_2 wafer and exposed to light as described in Section 2.3.1. The wafer was then incubated in 10 μg ml^{-1} TRITC labelled goat anti-rabbit IgG in PBS, for 60 minutes. After washing in PBS and distilled water the sample was dried in a stream of nitrogen and examined using fluorescent microscopy. Results are presented in Section 2.13.2.

2.3.2.1 Spectrophotometric determination of alkaline phosphatase activity.

Alkaline phosphatase (ALP) (E.C.3.1.3.1) activity was determined spectrophotometrically using p-nitrophenol phosphate (pNPP) as a substrate. pNPP is essentially colourless in solution, but when the phosphate is removed by the action of ALP the resultant p-nitrophenol gives an intense yellow colour with an absorbance maxima at 404 nm.

ALP was assayed by the addition to the sample of a 100 mM Tris-HCl buffer pH 9.8, containing 1mM $MgCl_2$ and 10 mM pNPP. Samples were incubated at room temperature for 15 minutes before reading the absorbance at 404 nm (proportional to ALP activity).

2.3.2.2. Assessment of cross reaction between photobiotin conjugates in solution.

To assess whether crosslinking occurred between the avidinated species-photobiotin complexes in solution, 2.5 ml of 20 μg ml^{-1} avidinated rabbit IgG was incubated with 2.5 ml of 20 μg ml^{-1} long arm photobiotin in PBS for 20 minutes. This solution was then split into two equal portions and half was exposed to light as it would be in the immobilisation procedure, whilst the other portion was kept in the dark. Both portions were then analysed using size exclusion chromatography using a 10 × 1 cm Sephadex G200 column, eluting

using 10 mM phosphate buffer pH 7.4. 1.0 ml fractions were collected and their protein content was determined by measurement of absorbance at 280 nm. The resultant elution profiles are shown in Section 2.13.2.

2.3.3 Method for light activated patterning of a protein on a silicon dioxide surface using an avidin / photobiotin / immunoglobulin construct

Avidin was immobilised on a SiO_2 wafer using the method described in Section 2.3.1 but replacing the BSA with avidin D. The avidin-modified wafer was incubated in 5.0 ml of 10 $\mu g\ ml^{-1}$ long arm photobiotin in PBS for 20 minutes, this and all subsequent stages were performed under dark room conditions. The wafer was covered with 10 $\mu g\ ml^{-1}$ rabbit IgG in PBS and exposed to light as described in Section 2.3.1. The temperature of the wafer was monitored throughout the exposure period and did not increase by any more than 5°C. The wafer was covered with 10 mg ml^{-1} casein in PBS and exposed to light for 15 minutes in the absence of a mask. The wafer was then incubated in 10 $\mu g\ ml^{-1}$ TRITC labelled goat anti-rabbit IgG in PBS, for 60 minutes. After washing in PBS and distilled water the sample was dried in a stream of nitrogen and examined using fluorescent microscopy. Results are presented in Section 2.13.3.

2.4 Method for Investigation into Achievable Resolution of Protein Patterning using Avidin-Photobiotin Technique.

The methodology of this Section of work was identical to that in Section 2.3.3 apart from the lithographic mask used, which consisted of series of lines (equal mark:space ratio) ranging from 1μm to 10μm in width. Results are presented in Section 2.14.

2.5 Investigation into Timescale of Avidin-Photobiotin Immobilisation Procedure.

2.5.1. Method for monitoring effect of exposure time on patterning process using fluorescence microscopy.

Avidin D and photobiotin were immobilised onto wafers of SiO_2 as described in Section 2.3.3. Wafers were then flood exposed to light from a mercury vapour lamp (irradiance ~ 9 mW cm^{-2}) in the presence of 10 mg ml^{-1} casein in PBS for varying periods of time. After washing in PBS all samples were exposed to light for 15 minutes through a photolithographic mask in the presence of 10 mg ml^{-1} chicken IgG in PBS. Following washing in PBS, samples were incubated with TRITC labelled rabbit anti - chicken IgG for one hour, and examined using fluorescence microscopy.

2.5.2. Method for monitoring effect of exposure time on patterning process using colourimetric enzyme assay.

Avidin D and photobiotin were immobilised onto wafers of SiO_2 as described in Section 2.3.3. Wafers were then flood exposed to light from a mercury vapour lamp (irradiance ~ 9 mW cm^{-2}) in the presence of 100 μg ml^{-1} ALP in PBS for varying periods of time. Other "control" samples were exposed to the enzyme for the same periods of time but were not

exposed to light, in order to monitor any variation in non-specific binding as a function of exposure time. After washing in TBS, the ALP activity of all samples was assayed as described in Section 2.3.2.1. The SiO_2 wafers were weighed and the enzyme activity related per gram of SiO_2 was calculated in order to correct for any variation in wafer size. The results are presented in Section 2.15.

2.6 Methods for Light Activated Patterning of Multiple Proteins onto Silicon Dioxide Surfaces

2.6.1 Method for light activated patterning of two immunoglobulins on a silicon dioxide surface using an avidin / photobiotin / immunoglobulin construct

Avidin and photobiotin were immobilised on a SiO_2 wafer using the silanisation process described in Section 2.3.1. The wafer was covered with 10 $\mu g\ ml^{-1}$ rabbit IgG in PBS and exposed to light through a mask with 10μm interval equal mark:space ratio lines as described in Section 2.3.1. The wafer was then covered with 10 $\mu g\ ml^{-1}$ rat IgG in PBS and exposed to light for 15 minutes through the same mask rotated through 90°. The wafer was covered with 10 mg ml^{-1} casein in PBS and exposed to light for 15 minutes in the absence of a mask. The wafer was then incubated in 10 $\mu g\ ml^{-1}$ TRITC labelled goat anti-rabbit IgG in PBS, for 60 minutes, washed and covered with 10 $\mu g\ ml^{-1}$ fluorescein isothiocyanate (FITC) labelled rabbit anti-rat IgG, for 60 minutes. After washing in PBS and distilled water the sample was dried in a stream of nitrogen and examined using fluorescent microscopy. A photomicrograph of this surface is shown in Section 2.16.1.

2.6.2 Method for patterning of five functional antibodies on a silicon dioxide surface using an avidin / photobiotin / immunoglobulin construct

Avidin (Neutravidin™ in place of Avidin D (note that the manufacturers of Neutravidin claim that it shows less non-specific binding than Avidin D, but Neutravidin was not available at the start of the study, hence the two type of avidin used)) and photobiotin were immobilised on a SiO_2 wafer using the silanisation process described in Section 2.3.1. The SiO_2 wafer was attached to a microscope so that it was in a fixed position below the microscope's objective lens with the top of the wafer level with the microscope stage. The five different antibodies were sequentially bound to the surface by selective light activation of distinct areas using a 100W high pressure mercury vapour lamp and a series of five photolithographic masks, each with a common registration mark (+) and a different number between 1 and 5 (50μm × 25μm in size) as a pattern. Polyclonal rabbit antibodies (10 μg ml^{-1}), raised against chicken, guinea pig, goat, mouse and rat were patterned in each case realigning the patterned substrate by means of the registration mark and a CCD camera attached to the microscope (the wafer was thoroughly washed in PBS between each exposure). The surface was then flood - exposed with light in the presence of 10 mg ml^{-1} casein in PBS, so immobilising the "blocking" protein at any previously unreacted photobiotin sites. After thorough washing with PBS, the wafer was incubated in a mixture of complementary antigens (chicken IgG, guinea pig IgG, goat IgG, mouse IgG and rat IgG, 100 μg ml^{-1} of each) in PBS at room temperature for one hour.

Following thorough washing with PBS, the wafer was incubated for one hour in 100 μg ml^{-1} rabbit anti - chicken IgG - TRITC in PBS and washed in PBS. Excess liquid was removed using a gentle stream of nitrogen, and the sample was examined using fluorescent

microscopy recording the image photographically (using a single lens reflex camera with automatic exposure times). This procedure was repeated sequentially, replacing the addition of rabbit anti - chicken IgG - TRITC with TRITC conjugates of anti - guinea pig IgG, rabbit anti - goat IgG, rabbit anti - mouse IgG or rabbit anti-rat IgG, on each occasion. Results are presented in Section 2.16.2.

2.7 Demonstration of Light Dependent Coupling of Proteins to Gold Surface

2.7.1 Method for demonstration of light dependent coupling of enzyme to gold surface

Avidin D was adsorbed on to two identical 2mm diameter gold electrodes (Bioanalytical systems (BAS)) by placing the electrodes in 5 ml of a 0.2mg ml^{-1} solution of avidin D in PBS for one hour at room temperature. After extensive rinsing with PBS the electrodes were then incubated in 5 ml of 10 $\mu g\ ml^{-1}$ solution of long arm photobiotin in PBS for 20 minutes under dark room conditions. After extensive rinsing with PBS electrodes were incubated with 50 µl of identical solutions of glucose oxidase (GOD) (50U ml^{-1}) in PBS, one electrode was retained in dark room conditions whilst the other was exposed to light from a high pressure mercury vapour lamp for 15minutes. After extensively rinsing both electrodes with PBS under dark room conditions, 50 µl of 10 mg ml^{-1} BSA in PBS was added to each electrode and both were exposed to light from a high pressure mercury vapour lamp for 15 minutes.

An amperometric enzyme based assay was performed using the modified gold surface as a working electrode, with an Ag/AgCl electrode as the reference and a bare platinum flag as a counter electrode. Chronoamperometry was performed in a working solution containing

10 mM sodium phosphate, 140 mM NaCl, 25 mM KCl, pH 7.4, or with the same solution but with the addition of 100 mM glucose. Initially, the working electrode was poised at a potential of 0V for 300 seconds, after which the potential was "stepped" to 650mV for 120 seconds during which time the current was monitored. Resultant traces and currents obtained 30 seconds after the application of the 650 mV potential are presented in Section 2.17.1.

2.7.2 Method for Demonstration of light dependent coupling of antibody to gold surface

Avidin D and photobiotin were immobilised onto two gold electrodes as described in Section 2.7.1. After extensive rinsing with PBS each electrode was incubated with 50 µl of 100 µg ml^{-1} rabbit anti-rat IgG in PBS whilst being exposed to light from a high pressure mercury vapour lamp for 15 minutes.

Electrodes were incubated in either 5 ml of 10 µg ml^{-1} rat IgG in PBS for 60 minutes, whilst the other was placed in 5 ml of 10 µg ml^{-1} rabbit IgG in PBS for 60 minutes. Following this both electrodes were extensively washed with PBS. Background currents were obtained by performing an amperometric measurement using the modified gold surface as a working electrode, with a Ag/AgCl electrode as the reference and a bare platinum flag as a counter electrode. After thorough rinsing, the electrodes were placed in 15ml of 25 mM KCl, 10mM hydrogen peroxide, 1 mM ferrocenemonocarboxylic acid. The potential was stepped to +150 mV for 120 seconds, during which time the current was monitored. After extensive washing with PBS the electrodes were incubated in 5 ml of a solution of 20 µg ml^{-1} horseradish peroxidase labelled rabbit anti-rat IgG at room temperature for 60 minutes, and

following thorough rinsing, amperometric measurements were made as described above. Results are presented in Section 2.17.2.

2.8. Method for Light Dependent Coupling of a Protein to a Glass Surface

A clean glass slide was immersed for 30 seconds in a 1% solution of 1,3-trimethoxysilyl-propylethylene diamine in 95% ethanol 5% distilled water, pH adjusted to 5.0 with glacial acetic acid. After removing the slide from this solution it was rinsed briefly in 95% ethanol 5% distilled water before being heated at 120°C for 30 minutes. The slide was immersed in 2% glutaraldehyde in PBS for 15 minutes, and then in a solution of 40mM sodium cyanoborohydride, 0.2mg ml^{-1} avidin D in PBS for 30 minutes at room temperature. After extensive rinsing with PBS the wafer was incubated in 5 ml of 10μg ml^{-1} long arm photobiotin in PBS for 20 minutes under dark room conditions. After extensive rinsing with PBS the wafer was covered with 10μg ml^{-1} rat IgG in PBS and exposed to light from a high pressure mercury vapour lamp for 15 minutes through a patterned chrome mask. Following further rinsing with PBS the wafer was covered with 10mg ml^{-1} BSA in PBS and the entire surface was exposed to light from the mercury vapour lamp for 15 minutes. The slide was incubated with 10μg ml^{-1} TRITC labelled rabbit anti-rat IgG in PBS, for 60 minutes at room temperature. After washing in PBS and distilled water the sample was dried in a stream of nitrogen and examined using a fluorescent microscope. A photomicrograph of the surface is shown in Section 2.18.

2.9. Method for Light Dependent Coupling of a Protein to a Carbon Surface

The carboxyl groups on two freshly polished glassy carbon electrodes (GCEs) were activated by the mixed anhydride method,[188] which allows primary amines in the avidin molecule to bind directly to activated carboxyl groups on the GCE surface. This procedure involved incubation of the GCEs in 10% HNO_3 for 10 seconds at +2V vs. Ag/AgCl followed by incubation in dimethylformamide (DMF) containing 1% N-methylmorpholine and 1.5% isobutylchloroformiate for 60 minutes (no electrical potential applied). The electrodes were then incubated in 50 mM sodium phosphate buffer pH 7.0 containing 100μg ml^{-1} avidin D for 2 hours. After extensive rinsing with PBS the electrodes were then incubated in 5 ml of a 10 μg ml^{-1} solution of long arm photobiotin in PBS for 20 minutes under dark room conditions.

After extensive rinsing with PBS each electrode had 50 μl of identical solutions of GOD in PBS aliquotted onto it. One electrode was subsequently retained in dark room conditions, whilst the other was exposed to light from a high pressure mercury vapour lamp for 15 minutes. After extensively rinsing both electrodes with PBS under dark room conditions, 50 μl of a 10 mg ml^{-1} solution of BSA in PBS was added to each electrode and both were exposed to light from a high pressure mercury vapour lamp for 15 minutes.

An amperometric enzyme based assay was performed using the modified carbon surface as a working electrode, with an Ag/AgCl electrode as the reference and a bare platinum flag as a counter electrode. Chronoamperometry was performed in a working solutions containing either 0 or 100 mM glucose solutions in 10 mM sodium phosphate, 140 mM NaCl, 25 mM KCl, pH 7.4. Initially, the working electrode was poised at a potential of 0V for 300

seconds, after which the potential was stepped to 650mV for 120 seconds during which time the current was monitored. Results are presented in Section 2.19

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2.10 Methods for Investigation into Reducing Non-Specific Binding on Gold Surfaces.

2.10.1 Method for investigation into reducing non-specific binding on gold surfaces.

Gold was evaporated onto a SiO_2 wafer as described in Section 3.5.1. The gold coated wafer was cut into portions of uniform size (10 × 4 mm), so that results obtained were directly comparable. 3 forms of avidin were examined, two deglycosylated preparations (Avidin D and Neutravidin) and the microbial type (streptavidin).

2.10.1.1 Immobilisation of avidin on gold by adsorption

The different forms of avidin were adsorbed onto gold by incubation of 100μg ml^{-1} avidin in 10 mM phosphate buffer (pH 7.0) for 16 hours at 4°C, followed by extensive washing in PBS.

2.10.1.2 Immobilisation of avidin on gold using NAC and EDC

Gold surfaces were incubated in 2 mM NAC in 10 mM phosphate buffer (pH 7.0) for 120 minutes at room temperature, followed by 120 minutes incubation in 1% (w/v) EDC in 10 mM phosphate buffer (pH 7.0). The gold surfaces were then incubated in 100μg ml^{-1} of the different forms of avidin in 10 mM phosphate buffer (pH 7.0) for 16 hours at 4°C

2.10.1.3 Immobilisation of avidin on gold using DTSSP

Gold surfaces were incubated in 2 mM DTSSP in 10 mM phosphate buffer (pH 7.0) for 120 minutes at room temperature. The gold surfaces were then incubated in 100μg ml^{-1} avidin in PBS for 16 hours at 4°C. As described in Section 2.1.8.2 the disulphide bond in DTSSP is thought to strongly chemisorb to the gold, and the NHS esters are available to react with primary amines in avidin to form a peptide bond.

2.10.1.4 Treatment with blocking agent and testing for non-specific binding.

Subsequent to the immobilisation of the avidin, the gold coated SiO_2 wafers were thoroughly washed in PBS and exposed to additional blocking proteins (BSA, casein and Superblock ™) by incubation in a 100μg ml^{-1} solution in PBS for 24 hours at 4°C. The gold coated SiO_2 wafers were then thoroughly washed in TBS (10 mM Tris, 140 mM NaCl, pH 7.4) and incubated in 10 μg ml^{-1} rabbit IgG - ALP in TBS for 15 minutes at room temperature. The samples were washed extensively in TBS and ALP activity was determined colourimetrically using pNPP as the substrate as described in Section 2.3.2.1. The absorbance was measured at 404 nm, and results were normalised against the response obtained for bare gold, the higher the response, the greater the degree of non-specific binding (note that the results were corrected to take account of the relatively small amount of non-specific binding occurring on the reverse side of the SiO_2 wafer by running controls of SiO_2 wafer that did not have gold attached). The results of these experiments are summarised in Section 2.20.

2.11 Methods to Investigate Regeneration of Avidinated Surfaces

2.11.1 Methods to investigate regeneration of avidinated silicon dioxide surfaces

Rabbit IgG and BSA were patterned (4μm equal mark:space ratio lines) onto SiO_2 surfaces as described in Section 2.3.3, and rabbit IgG was visualised by the addition of goat anti-rabbit IgG - TRITC conjugate. The surfaces were examined using fluorescence microscopy, and then underwent a number of treatments to ascertain if the avidinated surface could be regenerated.

The treatments were;

a, Incubation in NaOH solution pH 12 for 16 hours.

b, Incubation in HCl solution pH 1.5 for 16 hours.

c, Incubation in 6M guanidine in HCl solution pH 1.5 for 16 hours.

d, Incubation in 10M urea for 16 hours.

e, Incubation in PBS for 16 hours (control).

The surfaces were subsequently washed in distilled water, dried under a gentle stream of nitrogen and examined using fluorescence microscopy. The treated SiO_2 was then re-incubated with photobiotin and was subjected to repatterning with rat IgG and BSA as using the mask with 4μm equal mark:space ratio lines rotated through 90°. The presence of rat IgG was visualised by the addition of rabbit anti-rat IgG - FITC conjugate, and the samples were examined using fluorescence microscopy. The results are summarised in Section 2.21.1.

2.11.2. Method to investigate regeneration of avidinated gold surfaces

Four gold electrodes were incubated in 2 mM NAC in 10 mM phosphate buffer (pH 7.0) for 120 minutes at room temperature, followed by 120 minutes incubation in 1% (w/v) EDC in 10 mM phosphate buffer (pH 7.0). The electrodes were then incubated in 100μg ml^{-1} Neutravidin™ in 10 mM phosphate buffer (pH 7.0) for 16 hours at 4°C. All subsequent stages of the immobilisation procedure were performed at room temperature. After washing in PBS, the electrodes were first incubated in 10 mg ml^{-1} casein in PBS for 60 minutes and then in 10 μg ml^{-1} long arm photobiotin in PBS, for 20 minutes in the dark. All subsequent stages of the immobilisation procedure were performed in the dark. After extensive rinsing with PBS each electrode had 50 μl of identical solutions of goat anti-rat IgG in PBS placed onto it and the electrodes were exposed to light from a high pressure mercury vapour lamp for 15 minutes.

Two electrodes (#1 and #3) were incubated in 5 ml of 10 μg ml^{-1} rat IgG in PBS for 60 minutes, whilst the the others (#2 and #4) were incubated in 5 ml of 10 μg ml^{-1} rabbit IgG in PBS for 60 minutes. Following this electrodes were extensively washed with PBS. Background currents were obtained by performing an amperometric measurement using the modified gold surface as a working electrode, with a Ag/AgCl electrode as the reference and a bare platinum flag as a counter electrode. After thorough rinsing, the electrodes were placed in 15ml of 25 mM KCl, 10mM hydrogen peroxide, 1 mM ferrocenemonocarboxylic acid and a potential 150 mV was applied for 120 seconds during which time the current was monitored. After extensive washing with PBS the electrodes were incubated in 5 ml of a solution of 20 μg ml^{-1} HRP labelled goat anti-rat IgG at room temperature for 60 minutes, and following thorough rinsing, amperometric measurements were made as described

above. Currents obtained 30 seconds after the application of the 150 mV potential are shown in Section 2.21.2 (Table 2.6). Two (#1 and #2) electrodes were then incubated for 16 hours in 6M guanidine pH 1.5, whilst two (#3 and #4) were incubated for 16 hours in PBS (controls). After washing in PBS, the electrodes were incubated in 10 μg ml^{-1} long arm photobiotin in PBS, for 20 minutes in the dark. After extensive rinsing with PBS each electrode had 50 μl of identical solutions of goat anti-rabbit IgG in PBS placed onto it and the electrodes were exposed to light from a high pressure mercury vapour lamp for 15 minutes. The electrodes were then incubated with rat IgG or rabbit IgG, and HRP labelled goat anti-rabbit IgG and amperometric measurements were taken as previously described, the resultant currents are presented in Section 2.21.2.

2.12 Method for Characterisation of Biomolecular Patterns using Atomic Force Microscopy and Fluorescence Microscopy.

Neutravidin and photobiotin were immobilised on a SiO_2 wafer using the silanisation method described in Section 2.3.1. The wafer was covered with 10 μg ml^{-1} goat anti-rabbit IgG in PBS and exposed to light, as described in Section 2.3.1, for 15 minutes through a mask with 3μm interval equal mark:space ratio lines. The wafer was covered with 10 mg ml^{-1} casein in PBS and exposed to light for 15 minutes in the absence of a mask. The wafer was then incubated in 10 μg ml^{-1} TRITC labelled rabbit anti-rat IgG in PBS, for 60 minutes. After washing in PBS and distilled water the sample was dried in a stream of nitrogen and examined using fluorescent microscopy. A photomicrograph of this surface is shown in Section 2.22.

The pattern of antibodies was imaged using AFM, images were obtained using a Burleigh Personal AFM (Burleigh, UK) with Si tips (spring constant 0.045 nM m^{-1}, with a tip radius

of 10 nm and an aspect ratio of approximately 4:1, as quoted by Burleigh and verified using SEM (Hitachi S-900, Hitachi, Cambridge)). Imaging was carried out in air at ambient temperature and humidity with an imaging force maintained at approximately 5 nN. The resultant images are shown in Section 2.22.

2.13 Results and Discussion for Patterning of Proteins using Avidin and Photobiotin.

2.13.1 Results and discussion for patterning method using an avidin-protein conjugate by depositing a protein layer on the surface, adding photobiotin followed by the addition of the avidinated protein

The photomicrographs in Figures 2.14 and 2.16 (along with plots of relative fluorescent intensity in Figures 2.15 and 2.17) demonstrate that patterning of proteins was possible using this approach. However, the immobilisation efficiency (as evident by the intensity of the fluorescence and the lack of contrast between areas corresponding to mark and space on the photolithographic mask) was very low compared with other approaches (see Section 2.13.3). This may be due to steric hindrance of one or more of the coupling steps (i.e. the majority of photobiotin is bound to the immobilised protein layer, in such a way that the biotinyl groups are unavailable for subsequent binding to avidin). The data from the addition of the fluorescently labelled avidin suggested that the poor efficiency was due to the stage where avidin bound to the immobilised photobiotin, as again there was relatively little differentiation in fluorescent intensity in areas that had been exposed to light, and those that had not. A further disadvantage of this technique was that for a species to be immobilised it must have undergone prior avidination or biotinylation.

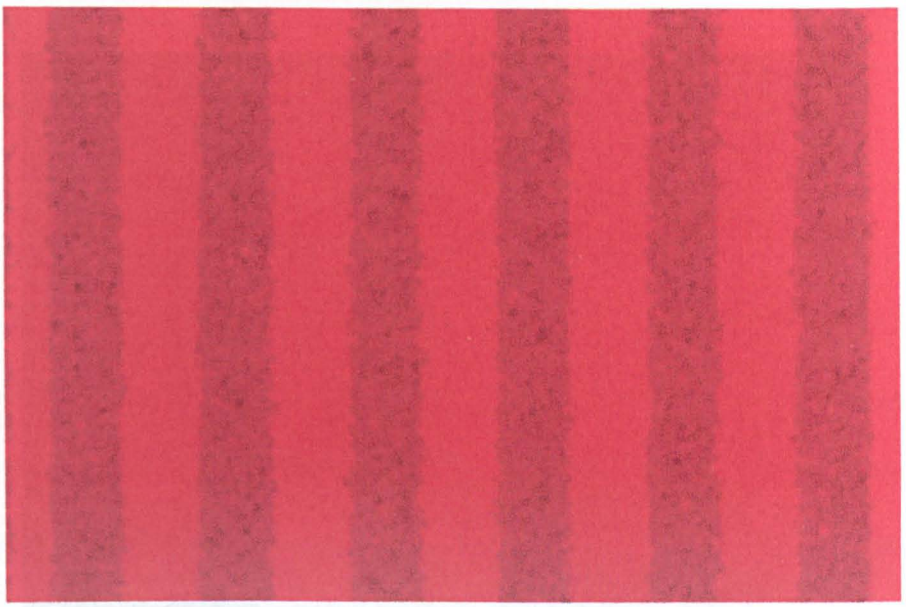


Figure 2.14 Photomicrograph of silicon dioxide patterned using a BSA / photobiotin / avidin / biotin-immunoglobulin construct visualised by a TRITC labelled anti-immunoglobulin antibody. BSA was immobilised on a SiO_2 wafer as described in Section 2.3.1. The BSA-modified wafer was incubated in a solution of long arm photobiotin in PBS whilst exposed to light as described in Section 2.3.1. The wafer was then incubated in avidin in PBS and after washing in PBS, the wafer was incubated with biotinylated rabbit IgG in PBS. The wafer was then incubated in TRITC labelled goat anti-rabbit IgG in PBS, and after washing in PBS and distilled water the sample was dried in a stream of nitrogen and examined using fluorescent microscopy.

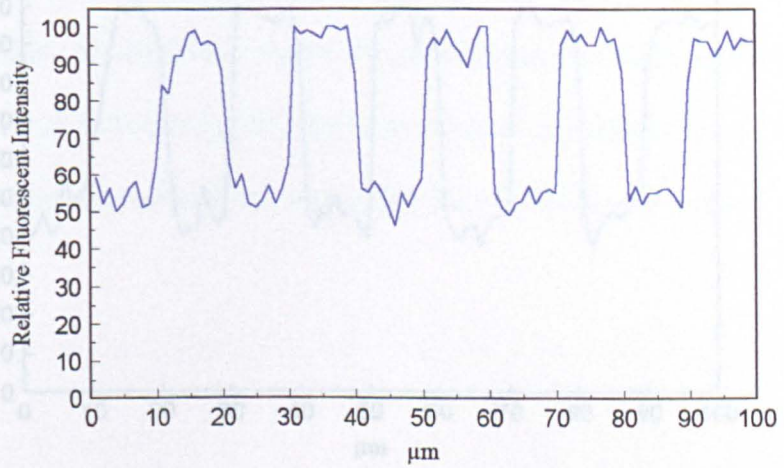


Figure 2.15 Plot of relative fluorescent intensity against distance for cross sectional profile through the pattern in Figure 2.14. The relative fluorescent intensity was determined by taking the brightest point (i.e. greatest fluorescence) on a line across the pattern as 100. A fluorescent intensity of 0 was set as a point that would show no fluorescence (i.e. totally black). The fluorescent intensity of each point on the line was determined by interpolation between these 2 extremes.

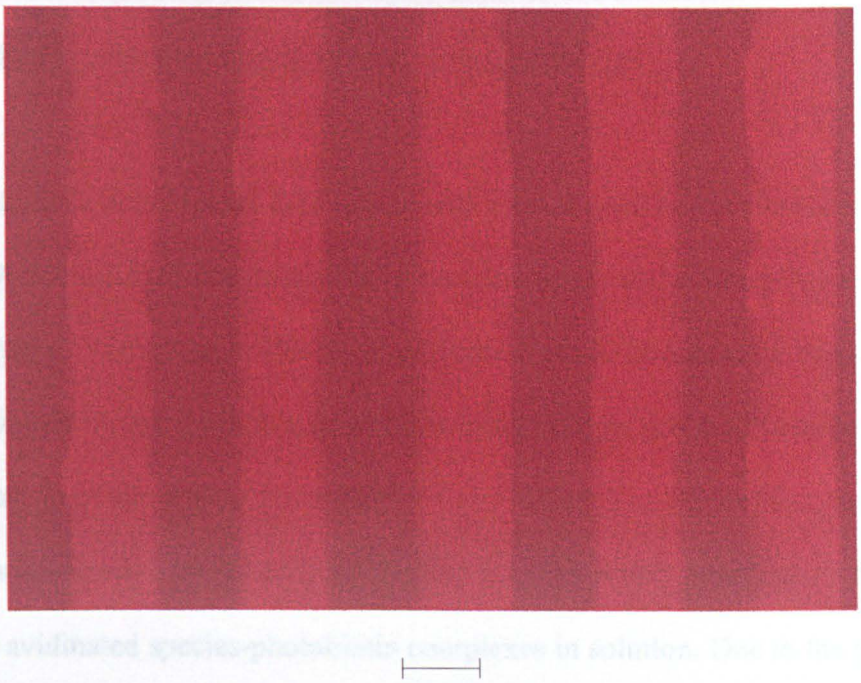


Figure 2.16. Photomicrograph of silicon dioxide patterned using a BSA / photobiotin / TRITC labelled avidin construct. BSA and photobiotin were immobilised as described in Section 2.3.1.1. The wafer was then incubated in TRITC labelled avidin in PBS, and after washing in PBS and distilled water the sample was dried in a stream of nitrogen and examined using fluorescent microscopy.

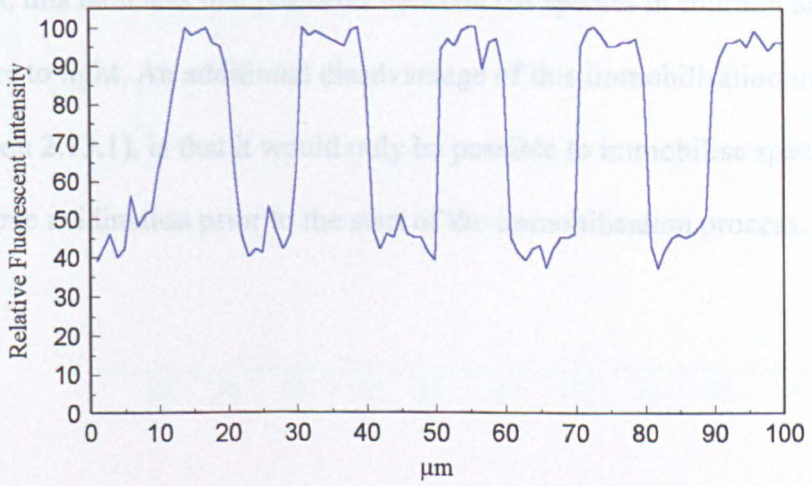


Figure 2.17 Plot of relative fluorescent intensity against distance for cross sectional profile through a section of the pattern in Figure 2.16. The relative fluorescent intensity was determined by taking the brightest point (i.e. greatest fluorescence) on a line across the pattern as 100. A fluorescent intensity of 0 was set as a point that would show no fluorescence (i.e. totally black). The fluorescent intensity of each point on the line was determined by interpolation between these 2 extremes.

2.13.2 Results and Discussion for patterning a protein by depositing a protein layer on the surface, and add avidinated protein with photobiotin attached

The photomicrograph in Figures 2.18 (along with plot of relative fluorescent intensity in Figures 2.19) demonstrate that patterning of proteins was possible using this approach. The immobilisation efficiency (as evident by the intensity of the fluorescence apparent when the wafer was viewed by eye through a fluorescence microscope, and the much longer exposure time necessary to obtain a photomicrograph) was low compared with other approaches (see 2.13.3). This appeared to be at least partially due to considerable crosslinking occurring between the avidinated species-photobiotin complexes in solution. Due to the generally faster reaction kinetics in solutions, it is likely that these complexes bound preferentially to each other rather than to the surface protein layer. Figure 2.17 shows the elution profile from size-exclusion chromatography of the material pre and post exposure to light. The increased A_{280} in the earlier fractions demonstrates that the post-exposure solution contained very high molecular weight material which was not present in the pre-exposure solution, this indicates that reactions between the species in solution had occurred upon exposure to light. An additional disadvantage of this immobilisation method (as described in Section 2.13.1), is that it would only be possible to immobilise species that had undergone avidination prior to the start of the immobilisation process.

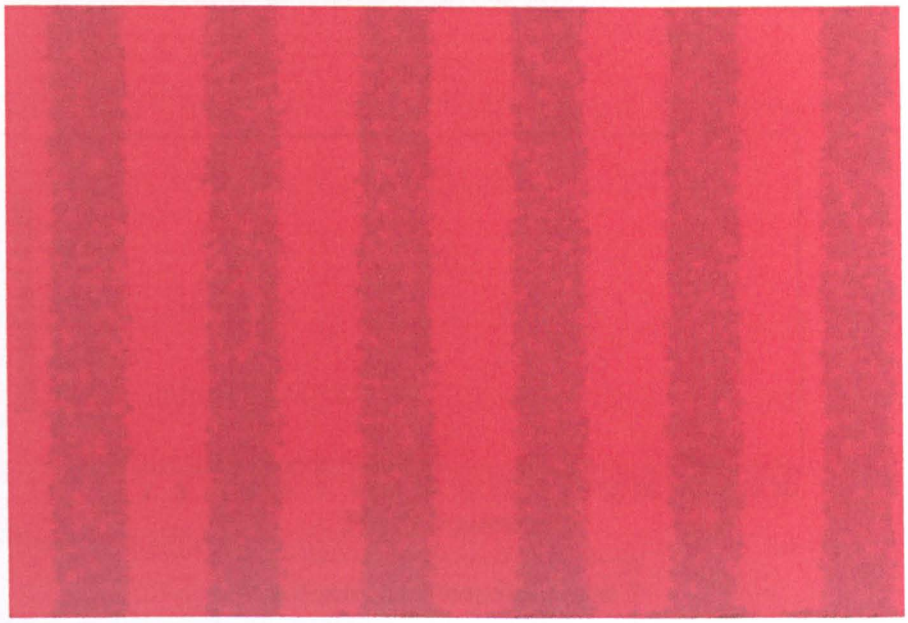


Figure 2.18 Photomicrograph of silicon dioxide patterned using a BSA / photobiotin-avidin - immunoglobulin construct. BSA was immobilised as described in scetion 2.3.2. Avidinated rabbit IgG was pre-incubated with long arm photobiotin in PBS, and was then placed on the protein coated SiO_2 wafer and exposed to light through a mask with 10µm interval equal mark:space ratio lines. The wafer was then incubated in TRITC labelled goat anti-rabbit IgG in PBS, and after washing in PBS and distilled water the sample was dried in a stream of nitrogen and examined using fluorescent microscopy.

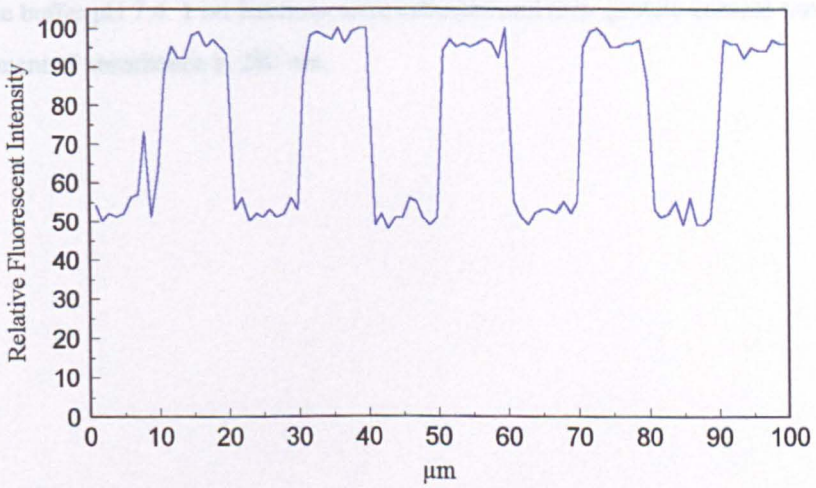


Figure 2.19 Plot of relative fluorescent intensity against distance for cross sectional profile through the pattern in Figure 2.18. The relative fluorescent intensity was determined by taking the brightest point (i.e. greatest fluorescence) on a line across the pattern as 100. A fluorescent intensity of 0 was set as a point that would show no fluorescence (i.e. totally black). The fluorescent intensity of each point on the line was determined by interpolation between these 2 extremes.

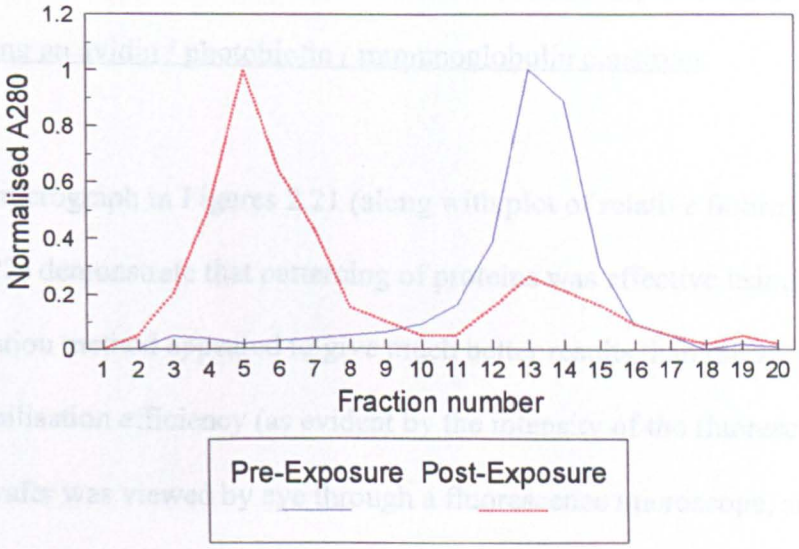
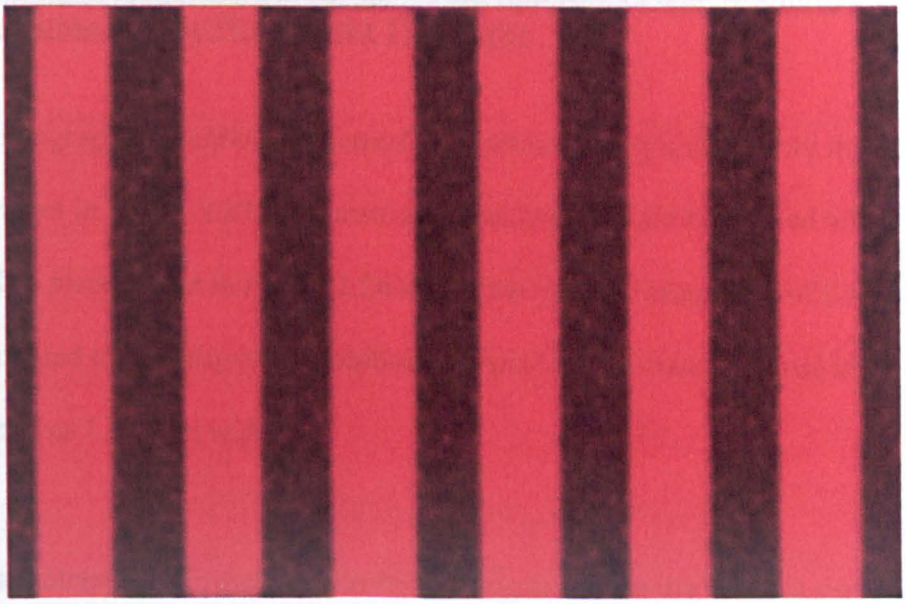


Figure 2.20 Normalised A_{280} from fractions from chromatographic separation of a photobiotin / avidin / protein solution, pre and post exposure to light. 2.5 ml of 20 μg ml^{-1} avidinated rabbit IgG was incubated with 2.5 ml of 20 μg ml^{-1} long arm photobiotin in PBS for 20 minutes. This solution was then split into two equal portions, and half was exposed to light for 15 minutes from a 100 W high pressure mercury vapour lamp sited 185 mm from the solution, whilst the other portion was kept in the dark. Both portions were then analysed using size exclusion chomatography using a 10 × 1 cm Sephadex G200 column, eluting using 10 mM phosphate buffer pH 7.4. 1 ml fractions were collected and their protein content was determined by measurement of absorbance at 280 nm.

2.13.3 Results and Discussion for light activated patterning of a protein on a silicon dioxide surface using an avidin / photobiotin / immunoglobulin construct

The photomicrograph in Figures 2.21 (along with plot of relative fluorescent intensity in Figures 2.22) demonstrate that patterning of proteins was effective using this approach. This immobilisation method appeared to give much better results than the alternative procedures. The immobilisation efficiency (as evident by the intensity of the fluorescence apparent when the wafer was viewed by eye through a fluorescence microscope, and the much shorter exposure time necessary to obtain a photomicrograph) was high compared with other approaches (see sections 2.13.1 and 2.13.2). The photomicrograph of the surface shown in Figure 2.21, and the plot (Figure 2.22) of relative fluorescent intensity against distance for cross sectional profile through the pattern showed that this immobilisation technique was capable of high resolution (<10μm) patterning of proteins onto a surface with low non-specific binding.



10 µm

Figure 2.21 Photomicrograph of silicon dioxide patterned with TRITC labelled IgG, using an avidin-photobiotin IgG construct as described in Section 2.3.3. The wafer was then incubated in TRITC labelled goat anti-rabbit IgG in PBS, and after washing in PBS and distilled water the sample was dried in a stream of nitrogen and examined using fluorescent microscopy.

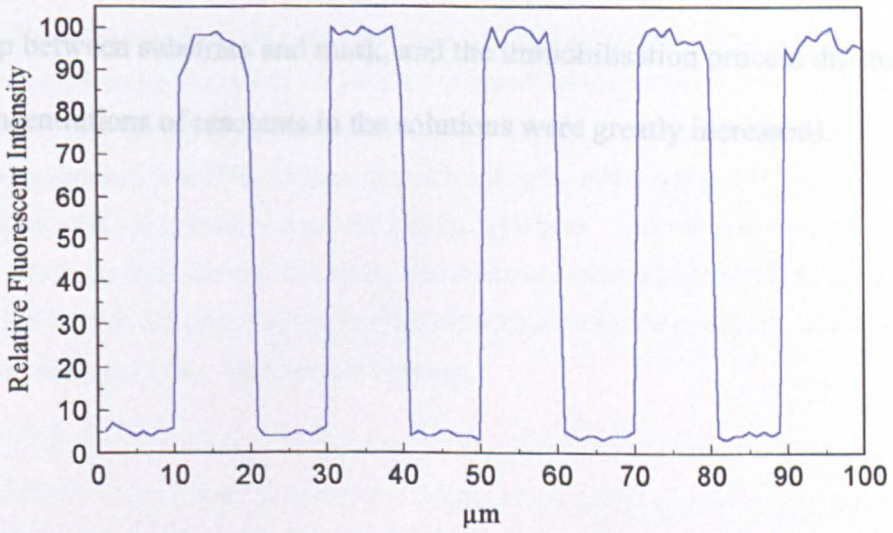


Figure 2.22 Plot of relative fluorescent intensity against distance for cross sectional profile through the pattern in Figure 2.21. The relative fluorescent intensity was determined by taking the brightest point (i.e. greatest fluorescence) on a line across the pattern as 100. A fluorescent intensity of 0 was set as a point that would show no fluorescence (i.e. totally black). The fluorescent intensity of each point on the line was determined by interpolation between these 2 extremes.

2.14 Results and Discussion for Investigation into Achievable Resolution of Protein Patterning using Avidin-Photobiotin Technique.

A photomicrograph of a surface patterned with protein using a mask with lines 1.5μm in width is shown in Figure 2.23, demonstrating that patterns can be formed with features of this size. The plots shown in Figure 2.24 show that baseline resolution of fluoresence (which is related to quantity of immobilised protein) is achieved for equal mark:space ratio lines as small as 1.5μm in width.

Although a pattern of fluorescent intensity relating to that on the mask is evident, baseline resolution is not achieved for lines 1 μm in width. The most likely explanation of the lack of resolution with 1 μm lines was that the mask was not in intimate enough contact to the substrate being patterned to overcome dispersion of the light through refraction etc. Attempts to achieve sub-micron resolution were not successful. Briefly, these experiments involved applying pressure to the mask to bring it in closer contact with the substrate to be patterned, however it was observed that by doing so the reactant solutions were forced out of the gap between substrate and mask, and the immobilisation process did not work (even when concentrations of reactants in the solutions were greatly increased).

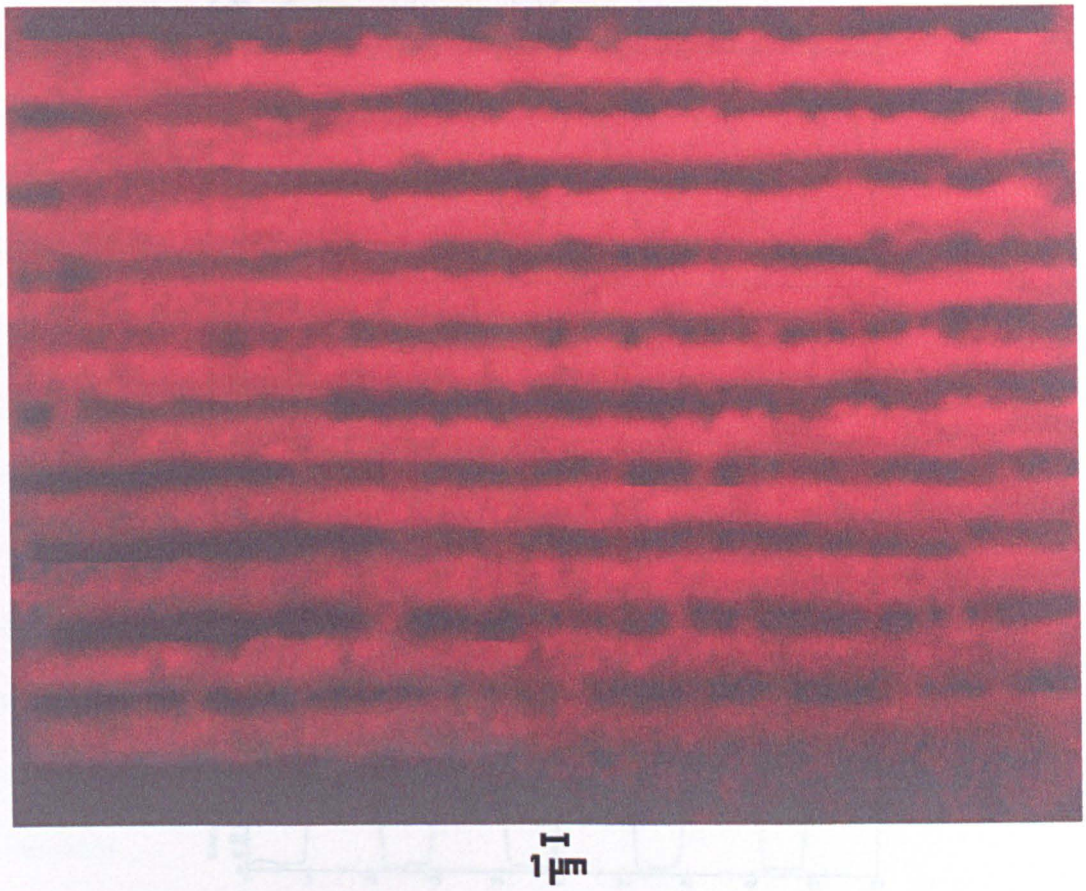


Figure 2.23 Photomicrograph of SiO_2 patterned with TRITC labelled IgG, using an avidin-photobiotin IgG construct. The photograph shows that 1.5 µm lines of immobilised protein can be patterned on a SiO_2 surface. Avidin was immobilised on a SiO_2 wafer as described in Section 2.3.3. The avidin-modified wafer was incubated with photobiotin in PBS, and was then covered with rabbit IgG in PBS and exposed to light through a mask with 1.5µm interval equal mark:space ratio lines. The wafer was covered with casein in PBS and exposed to light in the absence of a mask. The wafer was then incubated in TRITC labelled goat anti-rabbit IgG in PBS, and after washing in PBS and distilled water the sample was dried in a stream of nitrogen and examined using fluorescent microscopy.

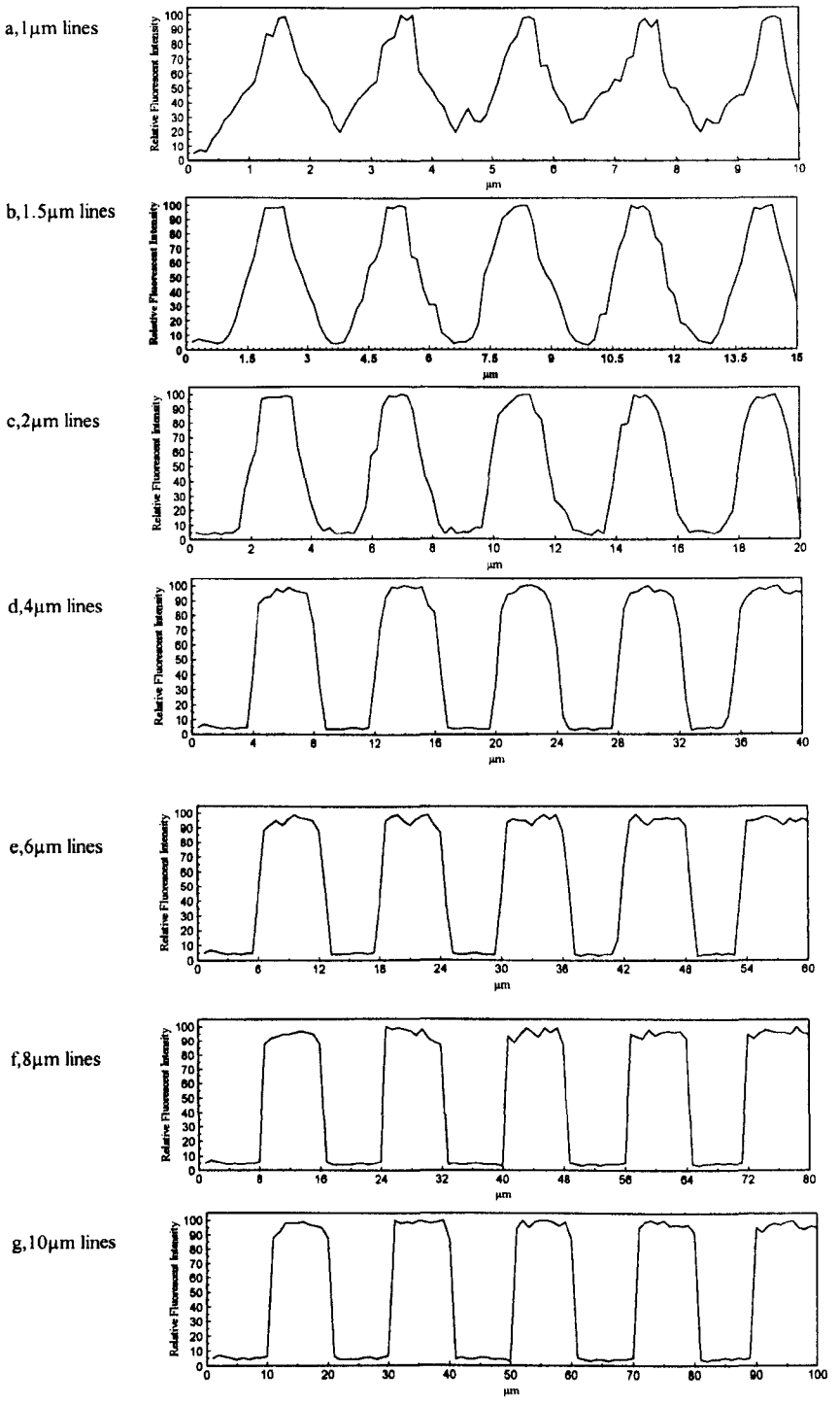


Figure 2.24 Plot of relative fluorescent intensity against distance for cross sectional profile of different width lines formed using the patterning of proteins as previously described. Proteins were patterned using avidin-photobiotin employing a photolithographic mask with equal mark:space ratio lines of different widths. The patterned proteins were visualised by the addition of fluorescently labelled antibodies, and resolution was examined by measurement of the relative fluorescent intensity across the pattern. The relative fluorescent intensity was determined relative to the brightest point (i.e. greatest fluorescence) assigned a value 100. A fluorescent intensity of 0 was set as a point that would show no fluorescence The fluorescent intensity of each point on the lines was determined by interpolation between these 2 extremes.

2.15 Results and Discussion for Investigation into Timescale of Avidin-Photobiotin Immobilisation Procedure.

2.15.1. Results and discussion for monitoring effect of exposure time on patterning process using fluorescence microscopy.

Upon fluorescence microscopy examination of surfaces treated as described in Section 2.5.1, patterned IgG was clearly visible when the substrate (with immobilised avidin and photobiotin) had been exposed to light in the presence of casein for up to 250 seconds prior to exposing the substrate to light in the presence of the IgG. When the substrate had been pre-exposed to casein and light for 500 seconds, only a very faint pattern corresponding to IgG could be observed, and no pattern could be discerned for pre-exposure times longer than 500 seconds. This data indicates that under the conditions described in Section 2.5.1, some of the photobiotin could still be activated following pre-exposure to light for up to 500 seconds.

2.15.2. Results and discussion for monitoring effect of exposure time on patterning process using colourimetric enzyme assay.

The results shown in Table 2.1 and Figure 2.25 indicate that under these experimental conditions (described in Section 2.5.2), the photobiotin mediated immobilisation process proceeds rapidly, and that it is close to completion (>90%) after 500 seconds. No further immobilisation occurs after 750 seconds. These results also demonstrate that the small amount of non-specific binding present also occurs rapidly, with approximately 60% of the material that is non-specifically bound being so in the first minute of exposure.

Time exposed to ALP (seconds)	ALP activity sample exposed to light (absorbance units / g SiO_2)	ALP activity sample kept in dark (absorbance units / g SiO_2)
0	0	0
1	1.1	0.8
10	8.2	1.2
25	17.1	1.5
50	31.4	1.8
100	51.6	2.2
250	66.9	2.4
500	71.4	2.8
750	75.3	3.0
1,000	74.9	2.9

Table 2.1 Enzyme activity versus time of exposure of avidin - photobiotin coated SiO_2 to an ALP solution in the presence or absence of light. Avidin D and photobiotin were immobilised onto wafers of SiO_2 as described previously. Wafers were then flood exposed to light in the presence of 100 μg ml^{-1} ALP in PBS for varying periods of time. Other "control" samples were exposed to the enzyme for the same periods of time but were not exposed to light. After washing in TBS, the ALP activity of all samples was assayed colourimetrically using pNPP as the substrate. Enzyme activity related per gram of SiO_2 was calculated.

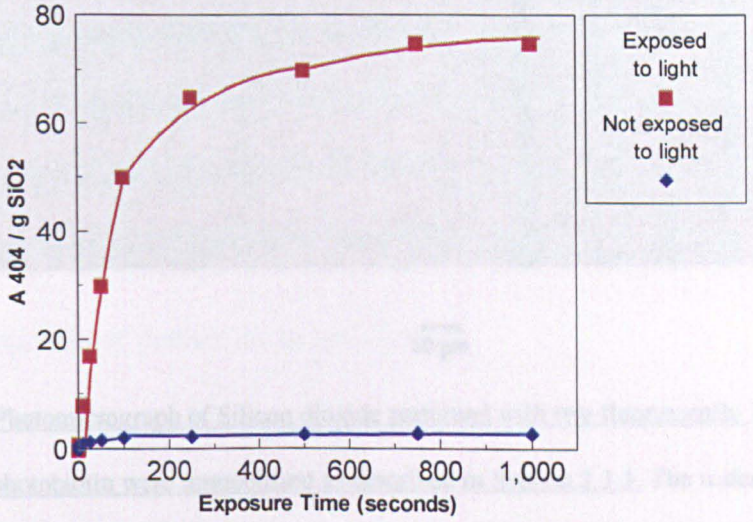


Figure 2.25 Plot of enzyme activity versus time of exposure of avidin - photobiotin coated SiO_2 to an ALP solution in the presence or absence of light. Avidin D and photobiotin were immobilised onto wafers of SiO_2 as described previously. Wafers were then flood exposed to light in the presence of 100 μg ml^{-1} ALP in PBS for varying periods of time. Other "control" samples were exposed to the enzyme for the same periods of time but were not exposed to light. After washing in TBS, the ALP activity of all samples was assayed colourimetrically using pNPP as the substrate. Enzyme activity related per gram of SiO_2 was calculated.

2.16 Results and Discussion for Light Activated Patterning of Multiple Proteins onto Silicon Dioxide surfaces

2.16.1 Results and discussion for light activated patterning of two immunoglobulins on a silicon dioxide surface using an avidin / photobiotin / immunoglobulin construct

Figure 2.26 demonstrates that the avidin / photobiotin technique can be used to pattern multiple proteins site specifically on to a silicon dioxide surface with minimal non-specific binding. It should be noted that in Figure 2.26 the TRITC lines are less red than those in for example Figure 2.24 owing to the fact that a double exposure (TRITC & FITC filters) was needed to obtain the former. This colour change was not due to non-specific binding of the FITC probe, as it occurs in the absence of the FITC label.

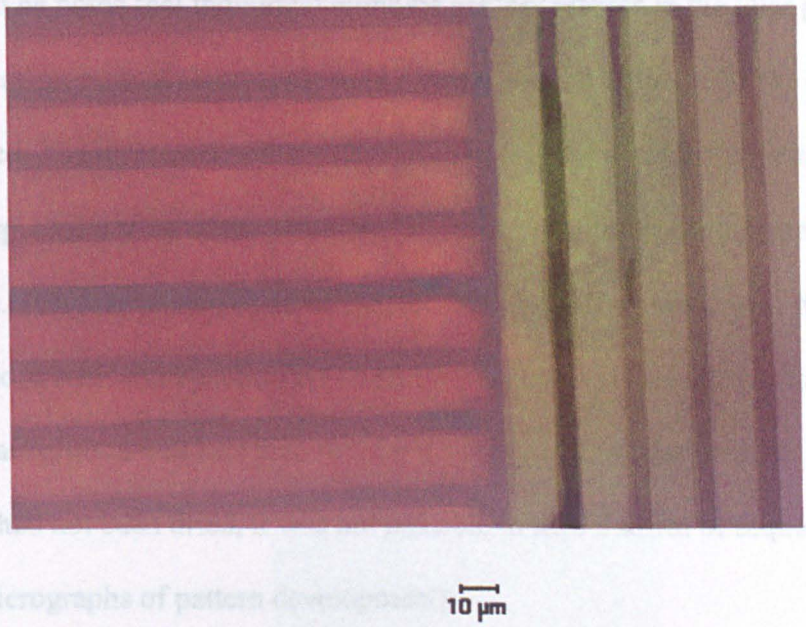


Figure 2.26 Photomicrograph of Silicon dioxide patterned with two fluorescently labelled antibodies. Avidin and photobiotin were immobilised as described in Section 2.3.3. The wafer was then sequentially incubated with rabbit IgG and rat IgG whilst exposed to light through a mask with 10µm interval equal mark:space ratio lines. In between the 2 incubations the mask was rotated through 90°. The wafer was then blcoked with casein and incubated in TRITC labelled goat anti-rabbit IgG and FITC labelled rabbit anti-rat IgG in PBS, and after washing in PBS and distilled water the sample was dried in a stream of nitrogen and examined using fluorescent microscopy.

2.16.2 Results and discussion for light activated patterning of five antibodies on a silicon dioxide surface using an avidin / photobiotin / immunoglobulin construct

Figure 2.27 shows a series of photomicrographs demonstrating the sequential patterning of different antibodies on a silicon dioxide surface. These photographs demonstrate the micrometre scale patterning of 5 functional antibodies (along with a sixth protein, casein) on to the surface (within an area of 200 × 50 μm). This work also demonstrates the construction of a qualitative multianalyte immunosensor, which is capable of specifically detecting 5 different antigens.

It should be noted that individual numbers are less intense in the later photomicrographs, as the overall amount of fluorescent light has increased and therefore the exposure time has decreased. The increased non-specific binding present in the later photomicrographs is due to the repeated drying of the substrate followed by addition of fluorescently labelled proteins. This was shown by repeating the experiment but not drying the sample in-between incubations with anti-species IgG - TRITC conjugates, where it was found that the intensity of the background was less than that for a sample that had been dried (note that as the sample had not been dried, it was not possible to take a series of sequential photomicrographs of pattern development).

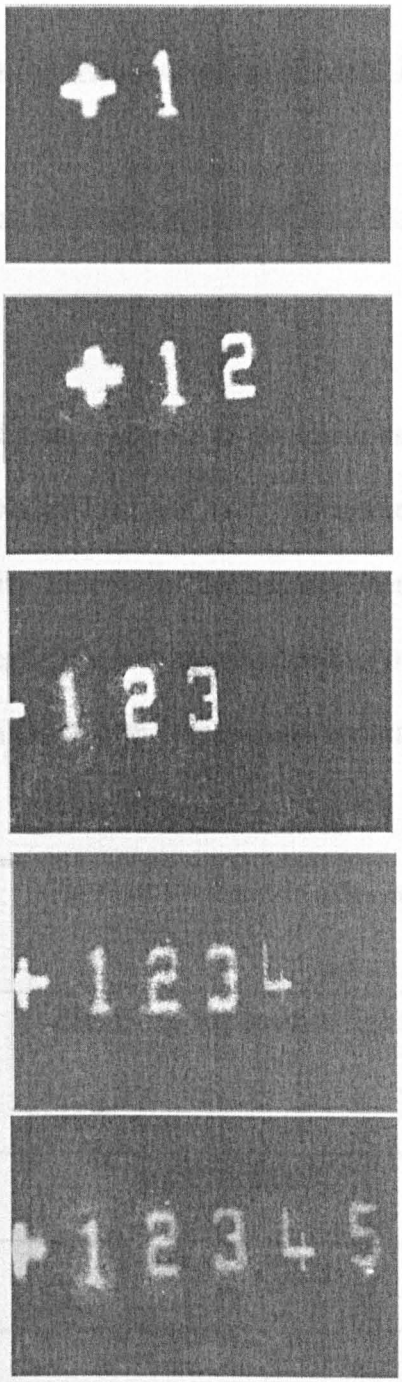


Figure 2.27 A series of fluorescence photomicrographs demonstrating the sequential patterning of different antibodies on a silicon dioxide surface. Polyclonal rabbit antibodies raised against chicken, guinea pig, goat, mouse and rat were patterned using the avidin-photobiotin immobilisation method, in each case realigning the patterned substrate by means of a registration mark and a CCD camera attached to the microscope. See Section 2.6.2 for experimental procedures.

2.17 Results and discussion for Demonstration of Light Dependent Coupling of Proteins to a Gold surface

2.17.1. Results and discussion for demonstration of light dependent coupling of an enzyme to a gold surface

The results shown in Table 2.2 and Figure 2.28 demonstrate that an enzyme (in this case, GOD) can be patterned on to a gold surface using the avidin photobiotin method, whilst retaining its biological activity. Electrodes that had not been exposed to light in the presence of GOD showed negligible non-specific binding of the enzyme, whilst electrodes that had been incubated with GOD in the presence of light showed a significant response to glucose.

	Current 30 seconds after application of 650 mV potential μA (± 1SD), n=5	
Glucose Concentration (mM)	**Electrode exposed to light**	**Electrode not exposed to light**
0	0.086 (±0.009)	0.089 (±0.010)
100	0.358 (±0.027)	0.084 (±0.006)

Table 2.2 Currents obtained from avidin - photobiotin coated gold electrodes which have been incubated with GOD in the presence or absence of light. Experimental details are in the text (Section 2.7)

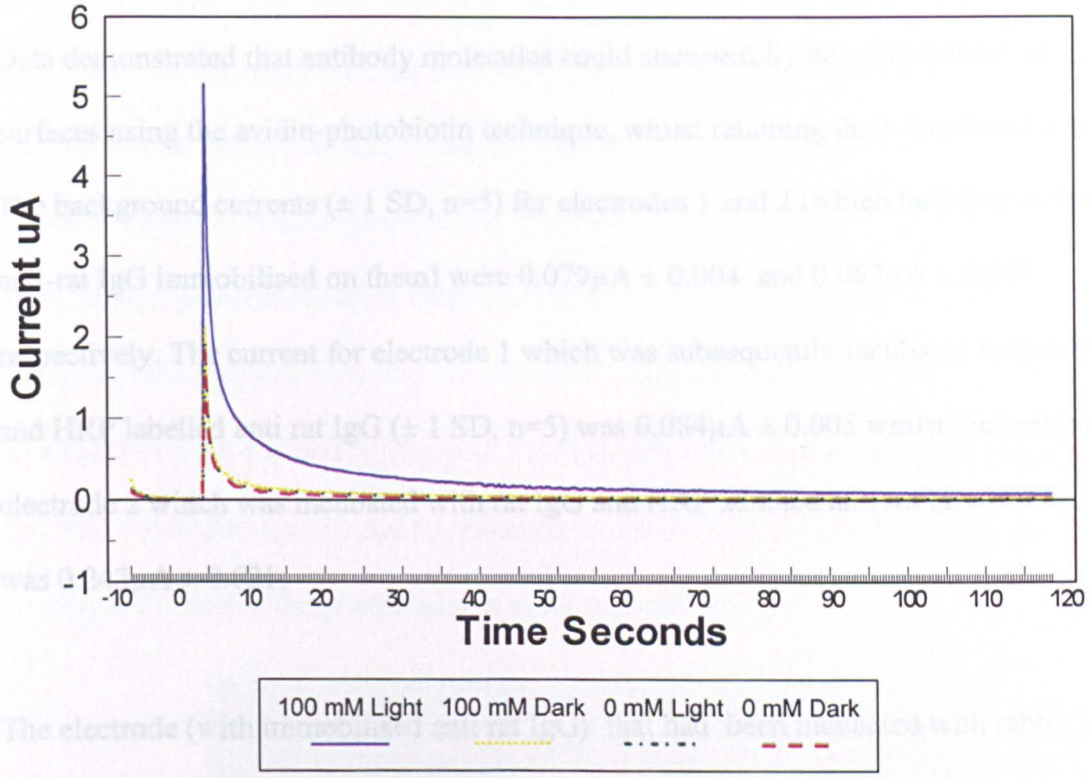


Figure 2.28 Chronoamperometric responses to glucose of avidin - photobiotin coated gold electrodes which have been incubated with GOD in the presence or absence of light. Experimental details are in the text (Section 2.7)

2.17.2 Results and discussion for demonstration of light dependent coupling of an antibody to a gold surface

Data demonstrated that antibody molecules could successfully be immobilised on to gold surfaces using the avidin-photobiotin technique, whilst retaining their functional activity. The background currents (± 1 SD, n=5) for electrodes 1 and 2 (which both had rabbit anti-rat IgG immobilised on them) were 0.079μA ± 0.004 and 0.083μA ± 0.005 respectively. The current for electrode 1 which was subsequently incubated with rabbit IgG and HRP labelled anti rat IgG (± 1 SD, n=5) was 0.084μA ± 0.005 whilst the current for electrode 2 which was incubated with rat IgG and HRP labelled anti rat IgG (± 1 SD, n=5) was 0.243μA ± 0.021.

The electrode (with immobilised anti rat IgG) that had been incubated with rabbit IgG showed negligible activity as demonstrated by the lack of response using the HRP-anti-rat IgG, whilst electrode that had been incubated with rat IgG showed a significant response. This work shows the application of the avidin - photobiotin immobilisation technique to the construction of a qualitative electrochemical immunosensor for the detection of rat IgG. This work was further developed later, as a quantitative immunosensor for FSH and LH (see Chapter 3).

2.18. Results and Discussion for Light Dependent Coupling of a Protein to a Glass Surface

For the patterning of proteins on a glass substrate, areas of fluorescence were observed which matched the mask that had been used (Figure 2.29), however this fluorescent image was not as well defined as when using the immobilisation procedure on SiO_2 surfaces, and the same degree of resolution could not be obtained. Individual lines could not be observed when using a photolithographic mask with equal mark:space ratio lines 10μm in width. A pattern of well resolved lines was clearly visible when using this mask and immobilisation procedure on SiO_2 substrates (Figure 2.21). The difference in resolution achieved using SiO_2 and glass substrates may be due to reflection of the incident light within the glass slide causing activation of the photobiotin in areas normally masked from light.

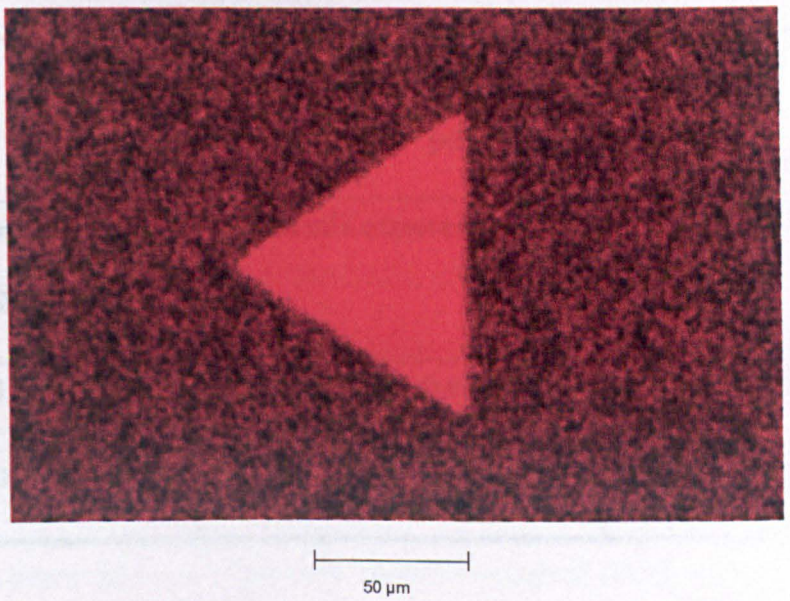


Figure 2.29 Photomicrograph of glass patterned with a fluorescently labelled antibody. Avidin was immobilised as decribed in Section 2.8. The avidin-modified slide was incubated with photobiotin in PBS, and was then covered with rat IgG in PBS and exposed to light through a mask with a triangular pattern. The wafer was covered with casein in PBS and exposed to light in the absence of a mask. The wafer was then incubated in TRITC labelled goat anti-rat IgG in PBS, and after washing in PBS and distilled water the sample was dried in a stream of nitrogen and examined using fluorescent microscopy.

2.19. Results and Discussion for Light Dependent Coupling of a Protein to a Carbon Surface

The results shown in Table 2.3 demonstrate that an enzyme (in this case, GOD) can be patterned on to a glassy carbon surface using the avidin photobiotin method, whilst retaining its biological activity. Electrodes that had not been exposed to light in the presence of GOD showed negligible non-specific binding of the enzyme, whilst electrodes that had been incubated with GOD in the presence of light showed a significant response to glucose. The currents for both of the electrodes in the 0 mM glucose, and that in 100 mM glucose for the electrode that had been incubated with GOD in the dark were very similar, whilst the current for 100 mM glucose with the electrode that had been incubated with GOD in the light was significantly increased.

	Current 30 seconds after application of 650 mV potential μA (± 1sd), n=5	
Glucose Concentration (mM)	**Electrode exposed to light**	**Electrode kept in dark**
0	0.092 (± 0.006)	0.102 (± 0.011)
100	0.275 (± 0.0232)	0.094 (± 0.010)

Table 2.3 Currents obtained from avidin - photobiotin coated glassy carbon electrodes which have been incubated with GOD in the presence or absence of light 30 seconds after application of 650 mV potential in glucose solutions. Experimental details are provided in the text (Section 2.9)

2.20 Results and Discussion for Investigation into Reducing Non-Specific Binding on Gold Surfaces.

It can be seen from Table 2.4 that the results obtained for Neutravidin and streptavidin are very similar, and non-specific binding tends to be lower with these preparations than with avidin D. The method of immobilisation of the avidin has a significant effect on the non-specific binding, with thiol mediated linkage (NAC & EDC, DTSSP) providing better results than that involving simple adsorption. A significant reduction in non-specific binding is seen with the use of all of the blocking agents, but the reduction is greater for BSA and casein than for Superblock. A low degree of non-specific binding is obtained when immobilising either Neutravidin or streptavidin to gold via a thiol, and using either BSA or casein as a subsequent blocking reagent

As has already been discussed, native avidin tends to show a high degree of non-specific binding of substances which is usually attributed to two inherent characteristics of the molecule, namely the high pI and the presence of carbohydrate residues.[185,191] Streptavidin which has a lower carbohydrate content, and deglycosylated avidin both exhibit reduced non-specific binding as compared to intact avidin.[185,191] The high isoelectric point (pI 10.0 - 10.5) of avidin, as well as the presence of carbohydrate (which accounts for about 10% of avidin's total molecular weight) on the protein contribute to non-specific binding in applications involving avidin. Due to its more favourable isoelectric point and lack of carbohydrate, streptavidin has historically been the accepted substitute for applications where the non-specific binding characteristics of avidin are unacceptable. More recently, modified forms of avidin such as Neutravidin have become available, with pI values closer to 7.0 and reduced carbohydrate contents leading to improved non-specific binding characteristics.[192,193]

	Normalised NSB % for blocking agent (± 1 sd), n=3			
Preparation of gold surface	No blocking reagent	BSA	Casein	Superblock™
Bare Gold	100 (±3.7)	5 (±0.3)	6 (±0.4)	23 (±2.7)
Gold + adsorbed avidin D	17 (±1.0)	3 (±0.3)	4 (±0.3)	5 (±0.4)
Gold + adsorbed Streptavidin	14 (±0.9)	4 (±0.4)	5 (±0.3)	7 (±0.5)
Gold + adsorbed Neutravidin	15 (±0.7)	3 (±0.3)	6 (±0.4)	8 (±0.6)
Au + avidin D linked by NAC & EDC	15 (±0.8)	6 (±0.3)	7 (±0.5)	9 (±0.5)
Au + Streptavidin linked by NAC & EDC	8 (±0.3)	3 (±0.3)	3 (±0.2)	5 (±0.3)
Au + Neutravidin linked by NAC & EDC	10 (± 0.4)	2 (±0.2)	3 (±0.3)	5 (±0.4)
Au + avidin D linked by DTSSP	13 (±1.0)	5 (±0.3)	4 (±0.2)	4 (±0.3)
Au + Streptavidin linked by DTSSP	9 (±0.7)	1 (±0.0)	3 (±0.2)	3 (±0.3)
Au + Neutravidin linked by DTSSP	7 (±0.5)	1 (±0.1)	2 (±0.2)	4 (±0.4)

Table 2.4 Non-specific binding to treated gold electrodes after 15 minutes exposure to IgG - ALP conjugate. Different avidin preparations and blocking agents were immobilised as summarised in the table. The gold electrodes were then washed in TBS and incubated in 10 $\mu g\ ml^{-1}$ rabbit IgG - ALP in TBS for 15 minutes at room temperature. The samples were washed extensively in TBS and ALP activity was determined colourimetrically using pNPP as the substrate.

2.21 Results and Discussion to Investigate Regeneration of Avidinated Surfaces

2.21.1 Results and discussion to investigate regeneration of avidinated silicon dioxide surfaces

As can be seen from the results in Figure 2.30 and Table 2.5, the patterned protein is resistant to treatment with HCl solution (pH 1.5) and 10 M urea solution (i.e. the pattern is still visible after treatment with these agents), although it should be noted that treatment with these solutions may denature the protein and render it non-functional. No change in the patterned protein is observed for the control treatment (incubation with PBS). Patterned protein is not resistant to treatment (i.e. the pattern is no longer visible after treatment) with NaOH solution (pH 12), but the inability to repattern the surface would indicate that a specific cleavage of the avidin photobiotin bond is not responsible. The high degree of non-specific binding observed when repatterning is attempted, suggests that this protocol either removes avidin and blocking proteins from the surface, or that it denatures them such that they no longer form an effective barrier to non-specific binding. Patterned protein is not resistant to treatment with 6M guanidine at low pH, and the fact that it is possible to repattern the surface demonstrates that it is the avidin photobiotin bond that is being broken. Treatment with guanidine has previously been shown to be able to break the avidin biotin bond.[218] The ability to break the avidin - photobiotin bond with the use of guanidine, means that it is possible to regenerate the avidinated surface and to repattern using photobiotin, which would be advantageous if one was using expensive sensor elements that one wished to reuse.

2.21 Results and Discussion to Investigate Regeneration of Avidinated Surfaces

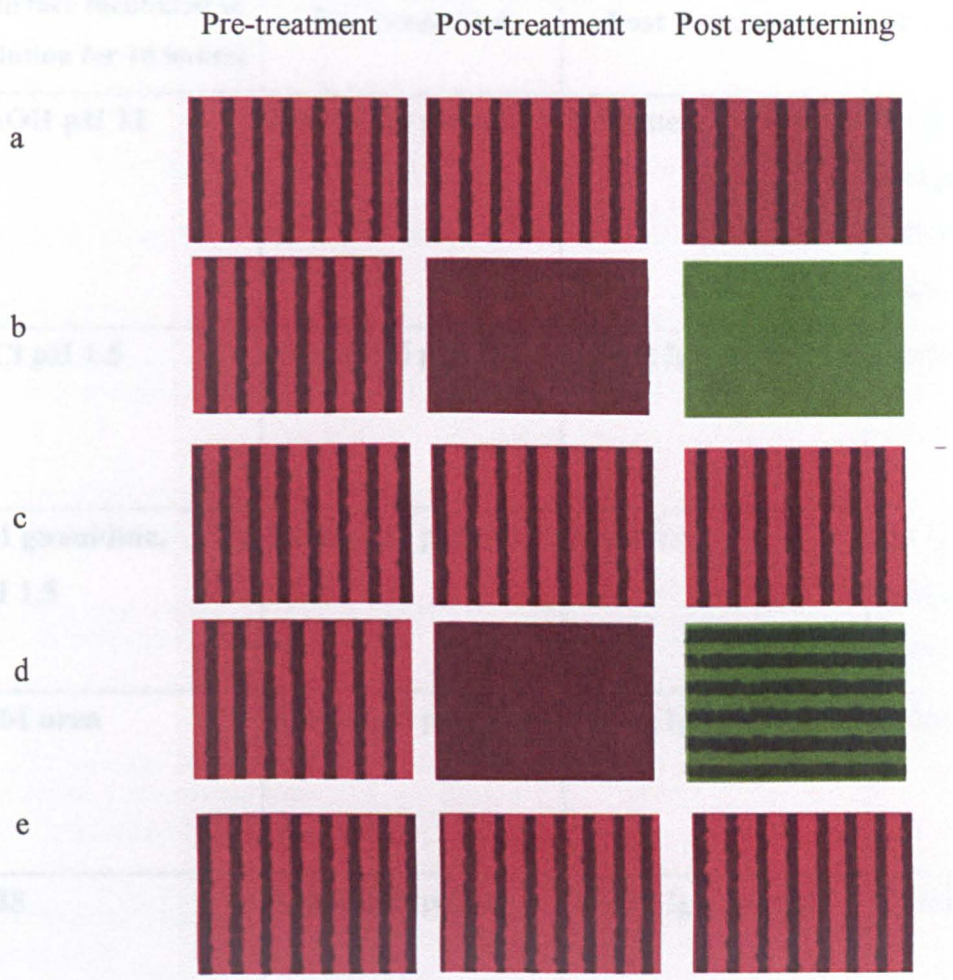


Figure 2.30 Fluorescent photomicrographs of attempts to regenerate avidinated SiO_2 surfaces. Patterns of rabbit IgG were generated on the surface using the avidin-photobiotin technique (detailed in Section2.11.1) The wafer was incubated in TRITC labelled goat anti-rabbit IgG in PBS, and examined using fluorescent microscopy. The surface then underwent a number of treatments; a) Incubation in NaOH solution pH 12. b) Incubation in HCl solution pH 1.5. c) Incubation in 6M guanidine in HCl solution pH 1.5. d) Incubation in 10M urea. e) Incubation in PBS (all for 16 hours). The surfaces were washed in water, dried under nitrogen and examined using fluorescence microscopy. The treated SiO_2 was re-incubated with photobiotin and subjected to repatterning with rat IgG and BSA as described previously. The presence of rat IgG was visualised by the addition of rabbit anti-rat IgG - FITC conjugate, and the samples were examined using fluorescence microscopy.

Treatment (surface incubated in solution for 16 hours)	Description of surfaces seen by fluorescence microscopy		
	Pre Treatment	Post Treatment	Post repatterning
NaOH pH 12	Rabbit IgG pattern	No pattern	No pattern. Bright background indicating high degree of NSB
HCl pH 1.5	Rabbit IgG pattern	Rabbit IgG pattern	Rabbit IgG pattern
6M guanidine, pH 1.5	Rabbit IgG pattern	No pattern	Rat IgG pattern. Indicates regeneration of avidinated surface.
10M urea	Rabbit IgG pattern	Rabbit IgG pattern	Rabbit IgG pattern
PBS	Rabbit IgG pattern	Rabbit IgG pattern	Rabbit IgG pattern

Table 2.5 Summary of results from procedures to attempt regeneration of avidinated surfaces. Patterns of rabbit IgG were generated on the surface using the avidin-photobiotin technique (detailed in Section 2.11.1) The wafer was incubated in TRITC labelled goat anti-rabbit IgG in PBS, and examined using fluorescent microscopy. The surface then underwent a number of treatments summarised in the table. The surfaces were washed in water, dried under nitrogen and examined using fluorescence microscopy. The treated SiO_2 was re-incubated with photobiotin and subjected to repatterning with rat IgG and BSA as described in Section 2.11.1. The presence of rat IgG was visualised by the addition of rabbit anti-rat IgG - FITC conjugate, and the samples were examined using fluorescence microscopy.

2.21.2 Results and discussion to investigate regeneration of avidinated gold surfaces

The data shown in Table 2.6 demonstrates that guanidine can be used to repattern proteins immobilised on gold surfaces using the avidin-photobiotin technique. The selectivity of the guanidine treated electrodes has changed, whilst that of the controls that were incubated in PBS has not.

Electrode Treatment	**Current (μA) 30 seconds after the application of 150 mV potential, for electrodes exposed to different antigens**			
	Electrode 1 (Rat IgG as Ag)	**Electrode 2** (Rabbit IgG as Ag)	**Electrode 3** (Rat IgG as Ag)	**Electrode 4** (Rabbit IgG as Ag)
None (Background current)	0.08	0.09	0.08	0.08
Incubation with antigen and HRP - anti rat IgG	0.31	0.10	0.31	0.09
Treatment with guanidine and repatterning	0.09	0.08	-	-
Treatment with PBS and repatterning (control)	-	-	0.30	0.09
Treatment with guanidine, repatterning , incubation with antigen and HRP - anti rabbit IgG	0.09	0.35	-	-
Treatment with PBS, repatterning , incubation with antigen and HRP - anti rabbit IgG (control)	-	-	0.32	0.11

Table 2.6 Currents obtained from gold electrodes pre and post repatterning antibodies using guanidine. Experimental details are provided in the text (Section 2.11). This data indicates that avidin -photobiotin modified gold surfaces can be regenerated using guanidine, thus allowing repatterning to take place.

2.22 Results and Discussion for Characterisation of Biomolecular Patterns using Atomic Force Microscopy and Fluorescence Microscopy.

Figures 2.33 and 2.35 demonstrate that protein patterns formed by use of the avidin-photobiotin immobilisation technique can be imaged using atomic-force microscopy. In Figure 2.34, the difference in the height between a peak (consisting of two IgGs assembled onto an avidin layer) and a trough (consisting of casein on avidin) can be estimated as approximately 7nm, which is less than would be expected on the basis of crystallographic data.[219] This may be explained by the random orientation the molecules are likely to take, alternatively the relatively large tip force necessary to obtain these images which is likely to distort the molecules significantly during imaging.[216,217] It should be noted that in Figure 2.32 (a cross-sectional profile through the fluorescent photomicrograph in Figure 2.31) no significance can be attributed to the arbitrary units for relative fluorescent intensity with respect to the height of the pattern in the z direction.

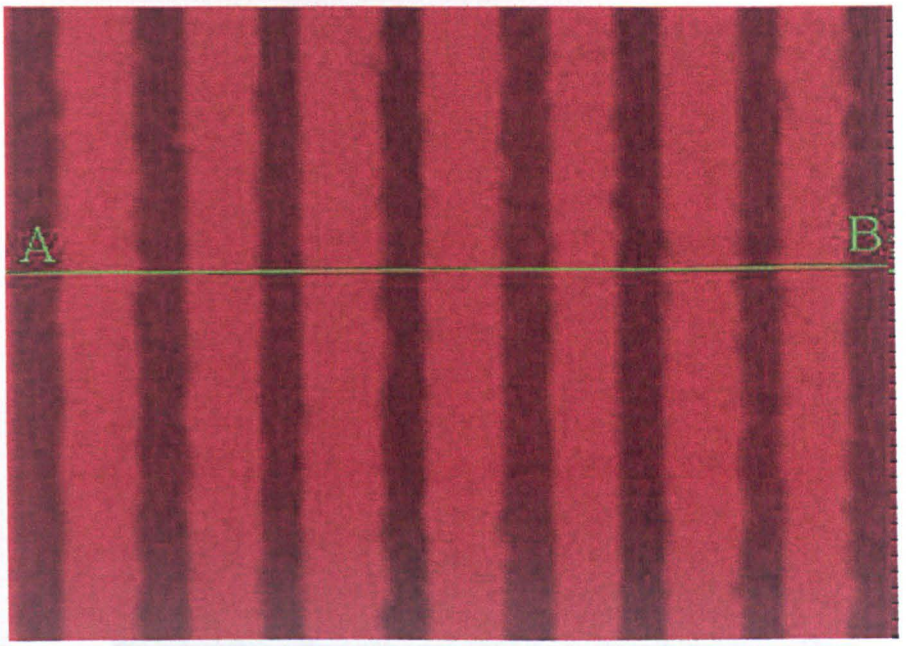


Figure 2.31. 2 - dimensional fluorescence micrograph of TRITC labelled IgG bound by a patterned complementary antibody. The patterning procedure is detailed in the text (Section 2.12). Following the protein immobilisation procedure, the wafer was incubated in TRITC labelled rabbit anti-rat IgG. After washing the sample was dried in a stream of nitrogen and examined using fluorescent microscopy.

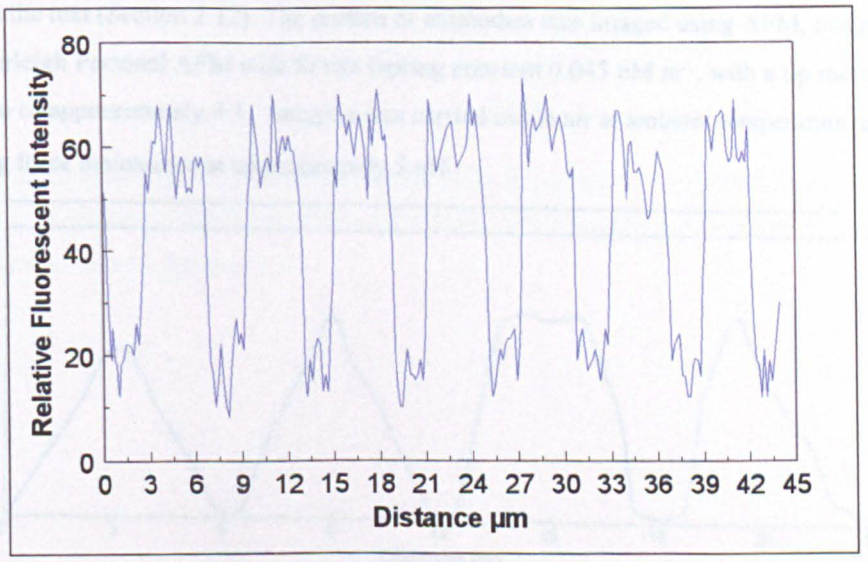


Figure 2.32 Cross sectional profile through the pattern between points A and B marked in Figure 2.31.

Patterned proteins were visualised by the addition of fluorescently labelled antibodies, and resolution was examined by measurement of the relative fluorescent intensity across the pattern. The relative fluorescent intensity was determined by taking the brightest point (i.e. greatest fluorescence) on a line across the pattern as 100. A fluorescent intensity of 0 was set as a point that would show no fluorescence (i.e. totally black). The fluorescent intensity of each point on the lines was determined by interpolation between these 2 extremes.

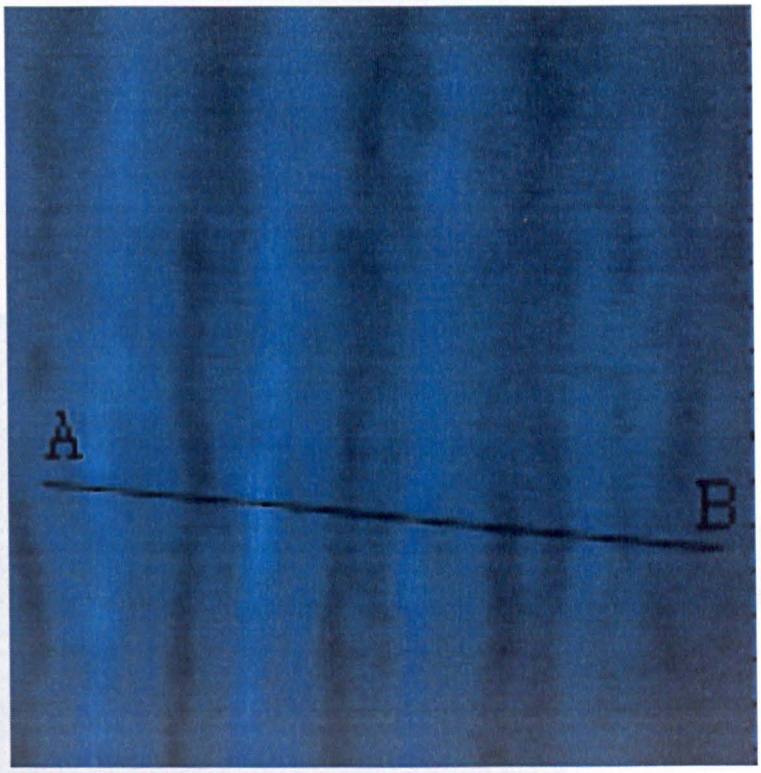


Figure 2.33. 2 - dimensional AFM image of the same pattern as in Figure 2.31. The patterning procedure is detailed in the text (Section 2.12). The pattern of antibodies was imaged using AFM, images were obtained using a Burleigh Personal AFM with Si tips (spring constant 0.045 nM m^{-1}, with a tip radius of 10 nm and an aspect ratio of approximately 4:1, Imaging was carried out in air at ambient temperature and humidity with an imaging force maintained at approximately 5 nN.

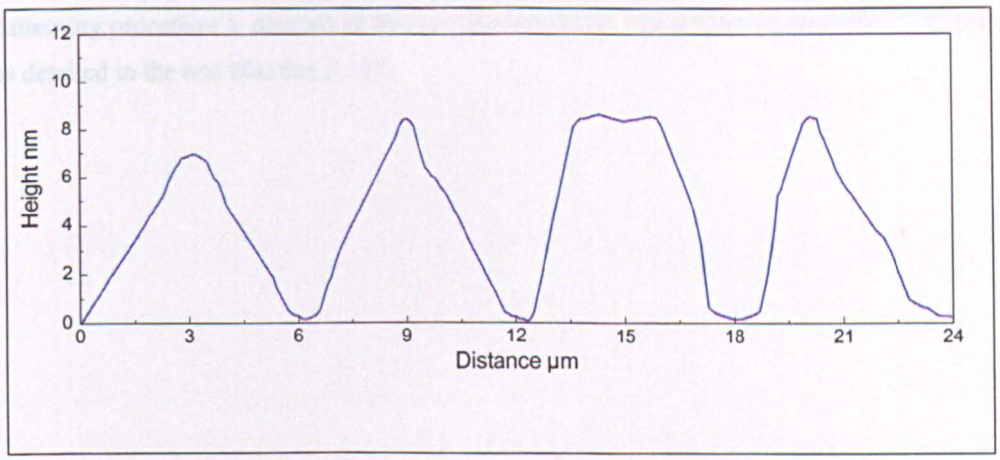


Figure 2.34. cross sectional profile through the pattern between points A and B marked in Figure 2.33. The plot was constructed from numerical data produced by the Burleigh AFM software.

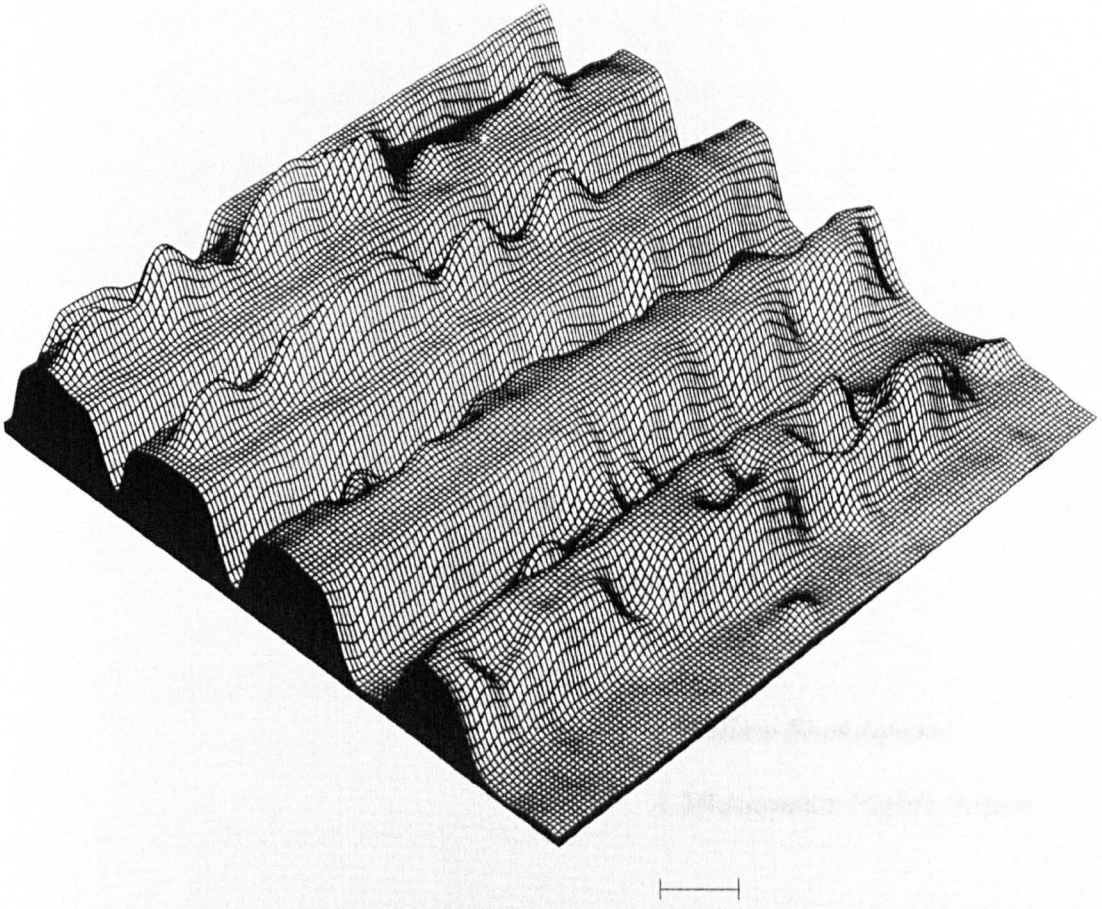


Figure 2.35 3-dimensional reconstruction from AFM data of an area of a protein patterned surface. The patterning procedure is detailed in the text (Section 2.12). The pattern of antibodies was imaged using AFM, as detailed in the text (Section 2.12).

CHAPTER 3

Question your desires,

Know of your youth,

Examine well your blood

William Shakespeare

A Midsummer Nights Dream

3 Simultaneous Determination of FSH and LH using a Multianalyte Immunosensor

The aim of the work described in this chapter of the thesis was to demonstrate the application of avidin-photobiotin technology by constructing a multianalyte immunosensor for the simultaneous assay for follicle stimulating hormone (FSH) and luteinising hormone (LH). In this respect the measurement of FSH and LH was an attractive model system, as in clinical analysis, the determination of these hormones is usually requested simultaneously. Additionally, both FSH and LH are normally assayed by heterogeneous immunoassay, thereby providing techniques which could be readily adapted to use with devices fabricated in the course of this work.

3.1 Follicle Stimulating Hormone and Luteinising Hormone

Follicle stimulating hormone (FSH) and luteinising hormone (LH) are both glycoprotein hormones, with relative molecular masses of approximately 34,000 and 28,500 respectively.[220] Both hormones contain α and β subunits, the α subunits are identical in the two hormones and consist of a single polypeptide chain containing 89 amino acids and two carbohydrate side chains. The amino acid sequences of the α subunits are also common with those of the other glycoprotein hormones, thyroid stimulating hormone (TSH) and human chorionic gonadotrophin (hCG).[221] The β chains of FSH and LH contain polypeptide chains of 118 and 115 amino acids, respectively, but with little homology, and the associated carbohydrate chains also differ,[222] the carbohydrate content of FSH is approximately 30% whilst that of LH is about 15%. The β side chain found in LH is very similar to that found in hCG.[222]

3.1.1 Physiological function of FSH and LH.

FSH and LH are present in both males and females, and are secreted by the anterior pituitary under the control of the hypothalamus.[222] Secretion is subject to negative and positive feedback at both the hypothalamus and the pituitary by the secretory products of the gonads. The hypothalamic control of FSH and LH release (Figure 3.1) is by means of a decapeptide, gonadotrophin releasing hormone (GRH).

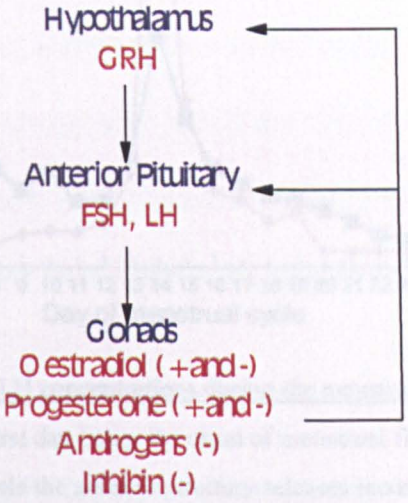


Figure 3.1 Schematic diagram illustrating production of gonadotrophin releasing hormone (GRH) by the hypothalamus which stimulates production of FSH and LH by the pituitary, which in turn stimulates the production of hormones by the gonadal tissue. Androgens and inhibin exert a negative feedback influence on the release of the gonadotrophins (LH and FSH), whereas oestradiol and progesterone can exert either a positive or negative feedback influence in females, dependent upon the time in the menstrual cycle.

Before puberty, FSH and LH production is low, but an early sign of puberty is fluctuating but sub-threshold secretion of both of these hormones. Once sustained secretion of supra-threshold hormonal levels occurs, gonadal growth in both sexes occurs. FSH and LH are responsible for the development of secondary sexual characteristics in both sexes, and for the onset of menstruation in females, these hormones are therefore referred to as

gonadotrophins. In females of reproductive age FSH and LH levels change during the course of the menstrual cycle as illustrated in Figure 3.2.

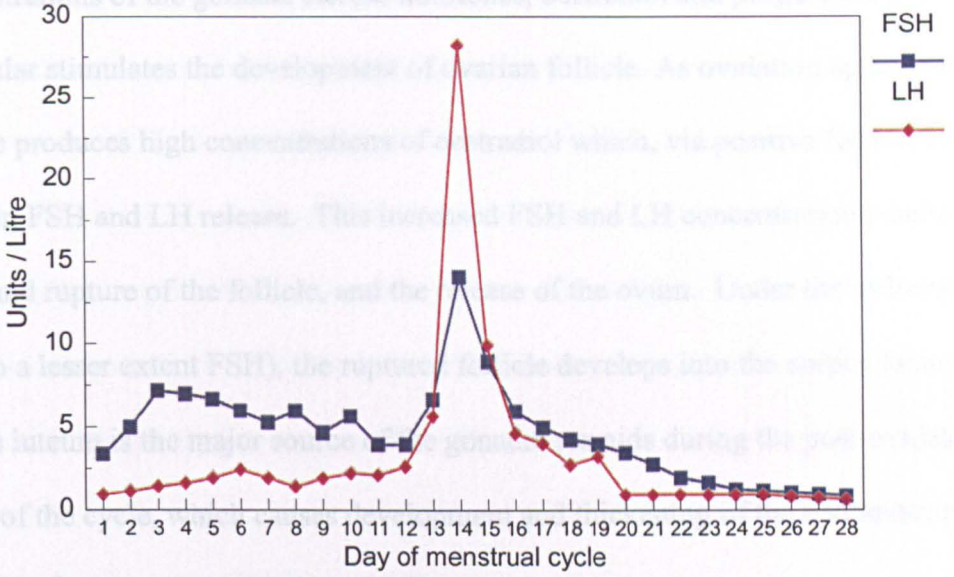


Figure 3.2 Typical plasma FSH and LH concentrations during the menstrual cycle.[223] The average length of the menstrual cycle is 28 days, the first day being the onset of menstrual flow, with ovulation occurring around day 14. At the start of the cycle the anterior pituitary releases increased quantities of FSH and LH in response to low circulating blood concentrations of oestradiol and progesterone. FSH in particular stimulates the development of ovarian follicle, as ovulation approaches the follicle produces high concentrations of oestradiol which, via positive feedback, cause a surge in FSH and LH release. This increased FSH and LH concentration results in rupture of the follicle, and the release of the ovum. Following ovulation oestradiol levels decrease, whilst progesterone levels increase reaching a maximum around day 21 of the cycle, at which time a second peak in oestradiol secretion occurs. The high circulating levels of progesterone and oestradiol inhibits the release of FSH and LH, the levels of which fall.

The average length of the menstrual cycle is 28 days, the first day being the onset of menstrual flow with ovulation occurring around day 14. At the start of the cycle the anterior pituitary releases increased quantities of FSH and LH in response to low circulating blood concentrations of the gonadal steroid hormones, oestradiol and progesterone. FSH in particular stimulates the development of ovarian follicle. As ovulation approaches the follicle produces high concentrations of oestradiol which, via positive feedback, causes a surge in FSH and LH release. This increased FSH and LH concentration results in a growth spurt and rupture of the follicle, and the release of the ovum. Under the influence of LH (and to a lesser extent FSH), the ruptured follicle develops into the corpus luteum. The corpus luteum is the major source of the gonadal steroids during the post-ovulatory (luteal) phase of the cycle, which causes development and thickening of the endometrium in readiness for pregnancy.

Following ovulation oestradiol levels start to decrease, whilst progesterone levels increase reaching a maximum at around day 21 of the cycle, at which time a second peak in oestradiol secretion occurs. The high circulating levels of progesterone and oestradiol inhibits the release of FSH and LH, and the corpus luteum which is reliant on LH for support decays. As the corpus luteum decays there is a marked decrease in oestradiol and progesterone secretion, and there is an associated sloughing and haemorrhage of the superficial layers of the endometrium and the cycle ends. If fertilisation does occur, the corpus luteum persists maintained by chorionic gonadotrophin (as already mentioned LH and hCG are very similar).

In males FSH stimulates the growth of the seminiferous tubules and maintains spermatogenesis, whilst LH promotes the secretion of androgens by the interstitial (Leydig) cells, hence LH's alternative name, interstitial cell stimulating hormone (ICSH).

Plasma FSH and LH levels increase with age in both sexes. In females this increase is associated with dwindling ovarian function, and the decreased effectiveness of negative feedback control by the gonadal steroid hormones. Upon the onset of the menopause atypical values are frequently measured, often with peak concentrations at the beginning and end of each cycle. Post menopausal plasma FSH and LH concentrations are increased and remain elevated.[221,222] Males do not show such an abrupt increase in levels, but older men tend to have a higher output of FSH.[224]

3.1.2 Diagnostic utility of FSH and LH

The determination of LH and FSH is of use in investigating male infertility. The majority of male infertility cases are eugonadotrophic with normal or slightly decreased LH and FSH levels, the infertility being a result of oligospermia due to failure of the seminiferous tubules. In hypergonadotrophic (primary) hypogonadism, the primary abnormality is in the testes. An increased LH indicates that there is failure of function of the Leydig cells, whereas an increased FSH indicates that there is a defect in the function of the Sertoli cells of the seminiferous tubules. In hypogonadotrophic (secondary) hypogonadism the primary abnormality is in the hypothalamus or pituitary, LH levels are low, and usually FSH levels are also decreased. The measurement of the gonadotrophins is important in being able to distinguish between hypergonadotrophic and hypogonadotrophic hypogonadism, as in both cases testosterone levels are low.[222,225]

The measurement of FSH and LH is most frequently used as a "fertility test" in women, or to differentiate between primary and secondary hypogonadism. Again in primary hypogonadism, the gonadotrophins will be increased due to negative feedback. Primary hypogonadism with a raised FSH and LH and low oestrogens is indicative of primary failure (which may be due to a chromosomal abnormality, autoimmune disease or idiopathic). An elevated LH with associated increased oestrogen levels, but a decreased or "low normal" FSH is indicative of polycystic ovary disease. In secondary hypogonadism low levels of FSH, LH and oestrogens are found, and in this case the abnormality is in the hypothalamus or pituitary or secondary to another endocrine disorder.[222,225]

The determination of LH and FSH is a "first line" test in the investigation of infertility in both males and females. Stimulation tests, where a stimulus is applied to the system and the resultant change in gonadotrophin levels is monitored, can be of use in the investigation of infertility for both sexes.[225]

LH levels, in particular, are frequently needed for immediate decision making for assisted conception techniques, and there is a need for reliable assays where results can be made available within two to three hours of sampling.[226] These tests are to determine when ovulation has occurred and to increase the chance of conception by monitoring the surge in gonadotrophin levels.

3.2 Review of Assay Methods Available for the Assay of FSH and LH

3.2.1 *In vivo* bioassays

A number of *in vivo* bioassays have been used for the determination of LH and FSH. LH has been measured by monitoring the weight increase of the ventral prostate of hypophysectomised immature male rats after administration of the sample preparation.[227] Similarly, the weight increase of the seminal vesicles in intact or hypophysectomised immature rats has also been used,[228,229] as the increase in weight is dependent upon the LH concentration in the sample. An alternative *in vivo* bioassay for LH was the ovarian ascorbic acid depletion assay. In this procedure immature female rats were pre-treated with pregnant mare's serum gonadotrophin and hCG. After the injection of the LH containing sample, the rat's ovaries were dissected and ascorbic acid, the concentration of which was related to the LH concentration, was determined.[230] FSH has been determined by monitoring the weight increase of rodent ovaries, when excess LH or hCG is administered simultaneously with the FSH containing test material.[231,232]

3.2.2 *In vitro* bioassays

LH has been assayed by monitoring the steroidogenic response of gonadal tissue. Either the production of progesterone by ovarian tissue [233,234] or testosterone by testicular interstitial cells[235-239] can be used, the progesterone or testosterone produced being assayed by radioimmunoassay. LH has also been determined by combining the ovarian ascorbic acid depletion test with histochemical techniques, the reducing activity of ovarian slices was assessed by staining with Prussian blue and performing microdensitometry.[240] The measurement of oestradiol production by cultured Sertoli cells provides an assay for FSH.[241]

In vitro radioreceptor assays exist for the assay of both LH[242-244] and FSH.[245,246] Radioreceptor assays depend on the competition for binding sites on gonadal tissue preparations, between the analyte in the test material and a tracer amount of radiolabelled hormone. The bound analyte and tracer are separated from the unbound fraction by centrifugation. The radioactivity in the bound fraction is inversely proportional to the concentration of the analyte in the test material.

3.2.3 Haemagglutination assays

The haemagglutination assay is a semi-quantitative test that is capable of distinguishing samples with very elevated analyte levels, from those normally encountered. The principle of the assay is that red blood cells are coated with antibodies raised against the analyte. Upon addition of the sample, the analyte is bound to the antibodies and therefore to the red blood cells. Each analyte molecule, has multiple antigenic sites, therefore when the substance being measured is in high concentration, "cross-linking" of the blood cells occurs and an agglutination pattern is observed which can be visually differentiated from normals.[247] The main advantages of haemagglutination assays are that they do not require specialist equipment, are relatively rapid to perform and are cheap. The major disadvantage is that they provide only very limited information, and will miss many cases which a fully quantitative assay would identify. However, haemagglutination assays for gonadotrophins have been developed, and are commercially available.[248]

3.2.4 Radioimmunoassay

FSH and LH have been measured by radioimmunoassay (RIA). This procedure uses radioisotopes as tracers to monitor the distribution of free and bound antigen in the presence of a known quantity of antibody. RIA depends on the competition for antibody binding sites between unlabelled antigen (*Ag*) in the sample and a known amount of added labelled antigen (*Ag**). As long as the antibody is not in excess, there is competition between the reactions in equations 3.1 and 3.2. The greater the amount of unlabelled antigen in the sample, the lower the amount of labelled antigen (and therefore, radioactivity) that is bound by the antibody (Figure 3.3). Separation of bound and free tracer has been achieved by centrifugation,[249-252] ethanol precipitation,[253] filtration[254] and electrophoresis.[255]

$$Ab+Ag \Leftrightarrow AbAg \quad (3.1)$$

$$Ab+Ag^* \Leftrightarrow AbAg^* \quad (3.2)$$

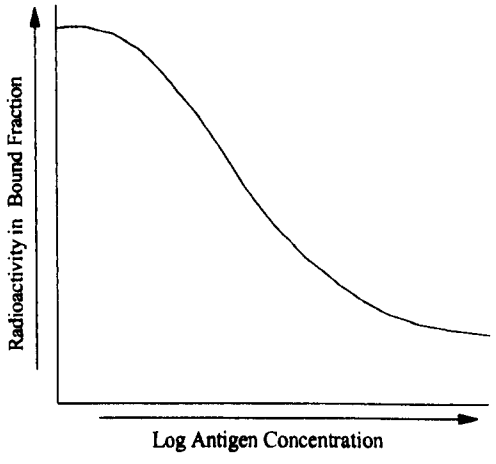


Figure 3.3 shows the semi-logarithmic dose response curve obtained in radioimmunoassay. This procedure uses radioisotopes as tracers to monitor the distribution of free and bound antigen in the presence of a limited quantity of antibody. RIA depends on the competition for antibody binding sites between unlabelled antigen in the sample and a known amount of added labelled antigen. The greater the amount of unlabelled antigen in the sample, the lower the amount of labelled antigen (and therefore, radioactivity) that is bound by the antibody.

Early RIA methods[249-252,254,255] used ^{131}iodine as the radiolabel, which decays by β and γ emission. The half life ($t_{½}$) of this isotope is 8.05 days and consequently it has a higher activity than ^{125} iodine, which decays with the production of γ radiation and has a half life of 60.2 days. The longer shelf life of ^{125}I, due to it's slower rate of decay, has meant that this is the most frequently used radiolabel in immunoassay.[256,257] Occasionally, the incorporation of iodine (a relatively large atom) into the structure of the antigen results in steric hindrance of the antibody-antigen reaction,[258] and in these cases ^{3}H is frequently used as the radiolabel (β emitter, $t_{½}$= 12.3 years).

Prior to the advent of monoclonal antibodies, great problems were encountered in obtaining specific antibodies that did not cross react with other glycoprotein hormones.[253] In most immunoassays for LH there was considerable cross-reactivity with hCG,[222] due to their structural similarities. This cross reactivity was exploited in early RIA methods for LH where antibodies against hCG were used as the basis of the assay, or more commonly radiolabelled hCG was used for the tracer and unlabelled hCG was used for standards.[249,254] Numerous RIA methods have been developed for the determination of LH and FSH levels and are commercially available.[257,260-262]

Radioimmunoassay is generally labour intensive and time consuming, although in recent years automated sample processing systems have reduced the labour required, and the time taken to obtain a result,[263-265] however they still require operation by highly trained staff.

3.2.5 Immunoradiometric assay

Immunoradiometric assay (IRMA)[266] is a non-competitive technique where an excess of radiolabelled antibody against the analyte is added to the sample. Separation of free and antigen-bound radiolabelled antibody is performed by the addition of a second antibody (immobilised on a solid phase) which is also directed against the antigen. After removal of the radiolabelled antibody by washing, the quantity of radiolabelled antibody bound to the solid phase is proportional to the antigen concentration in the sample (Figure 3.4). Due to the low background activity, IRMA techniques tend to have lower detection limits than their RIA counterparts. Numerous IRMA methods have been developed for the determination of LH and FSH levels and are commercially available.[261,262, 267-271]

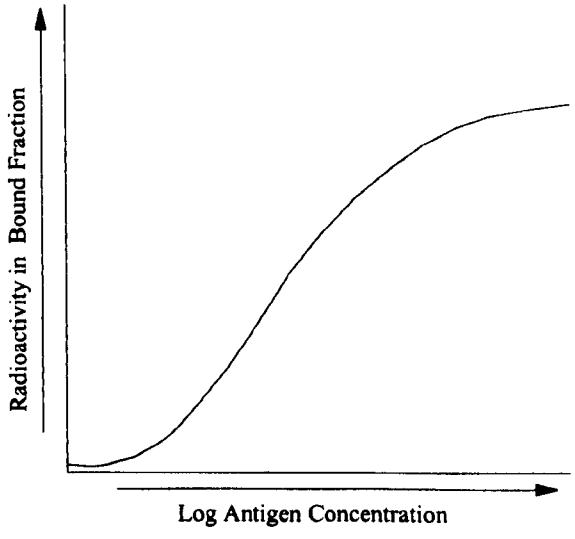


Figure 3.4 Semi-logarithmic dose response curve of an immunoradiometric assay. Immunoradiometric assay is a non-competitive technique where an excess of radiolabelled antibody against the analyte is added to the sample. Separation of free and antigen-bound radiolabelled antibody is performed by the addition of a second antibody (immobilised on a solid phase) which is also directed against the antigen. After removal of excess radiolabelled antibody by washing, the quantity of radiolabelled antibody bound to the solid phase is proportional to the antigen concentration in the sample.

3.2.6 Dual Radioisotopic assay

γ Radiation from different radioisotopes display different energy spectra, therefore it is possible to measure 2 analytes simultaneously by differentiating radioactivity arising from two different labels.[272] These types of assay are most appropriate for the measurement of those analytes that are measured in the same sample such as FSH / LH, TSH / thyroxine and Vitamin B_{12} / folate.[257]

3.2.7 Non-Isotopic immunoassays

As has already been discussed, in recent years there has been a move away from the use of radioactive labels, and they have largely been replaced by amongst others luminescent,[273] fluorescent,[93] and enzymatic labels.[94,95] These non-isotopic labels possess a number of advantages such as safety, longer shelf life, shorter assay time, easier automation, improved detection limits and they introduce the possibility of developing disposable devices that can be used to perform near-patient testing.

3.2.7.1 Enzymatic immunoassay

Enzymes are the most widely used labels in immunoassays, the reactions can be followed colourimetrically, fluorimetrically, electrochemically, calorimetrically, or by using luminescence. Like other immunoassays, those using enzymes can be classified into two types, heterogeneous and homogeneous. In heterogeneous enzyme immunoassay, after the antibody and antigen have been incubated together, a step is required to separate the free antigen and antibody from the antibody-antigen complexes. The enzymic activity of one or both of these fractions is then assessed. The most common heterogeneous enzyme

immunoassay is enzyme-linked immunosorbent assay (ELISA). In this technique either the antigen or the antibody can be labelled with the enzyme. In the so called 'Sandwich' ELISA assay, an excess of immobilised antibody is incubated with the sample and the antigen binds with the immobilised antibody. After washing away the unbound sample constituents the immobilised antibody-antigen complex is incubated with excess enzyme-labelled antibody. This enzyme labelled antibody binds to a second antigenic site and excess enzyme-labelled antibody is washed off, therefore the remaining enzyme activity is directly proportional to the antigen concentration. A typical ELISA dose response curve is shown in Figure 3.5

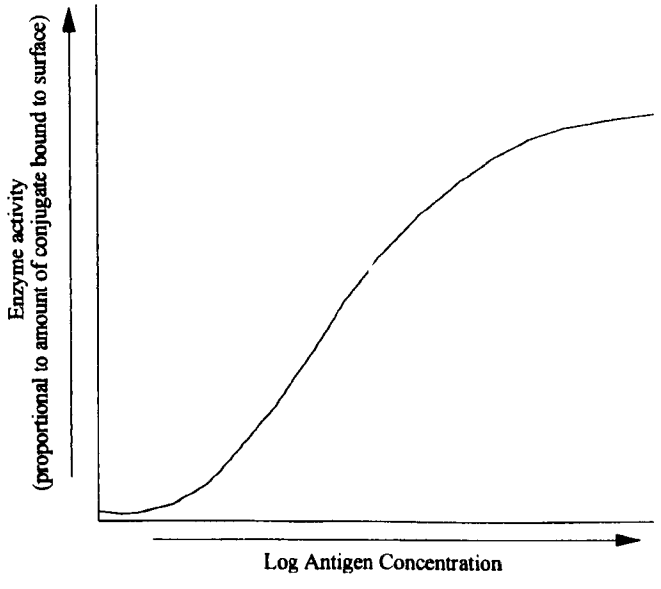


Figure 3.5 Typical semi-logarithmic dose response curve for a "sandwich" ELISA. In this technique an excess of immobilised antibody is incubated with the sample and the antigen binds with the immobilised antibody. After washing away the unbound sample constituents the immobilised antibody-antigen complex is incubated with excess enzyme-labelled antibody. This enzyme labelled antibody binds to a second antigenic site and excess enzyme-labelled antibody is washed off, therefore the remaining enzyme activity is directly proportional to the antigen concentration.

In homogeneous enzyme immunoassays (enzyme multiplied immunoassay technique (EMIT)), a separation process is not required, as the antigen-coupled enzyme activity changes when bound to an antibody. When the sample is added to this system, less of the antigen-enzyme conjugate will bind to the antibody, as it is in competition for the antibody binding sites with free antigen from the sample, therefore the change in enzymic activity is inhibited. A typical EMIT dose response curve is shown in Figure 3.6. ELISA is generally applicable to the measurement of almost any antigen, and can detect substances at much lower concentrations than can EMIT largely due to the lower inherent background signal for ELISA.

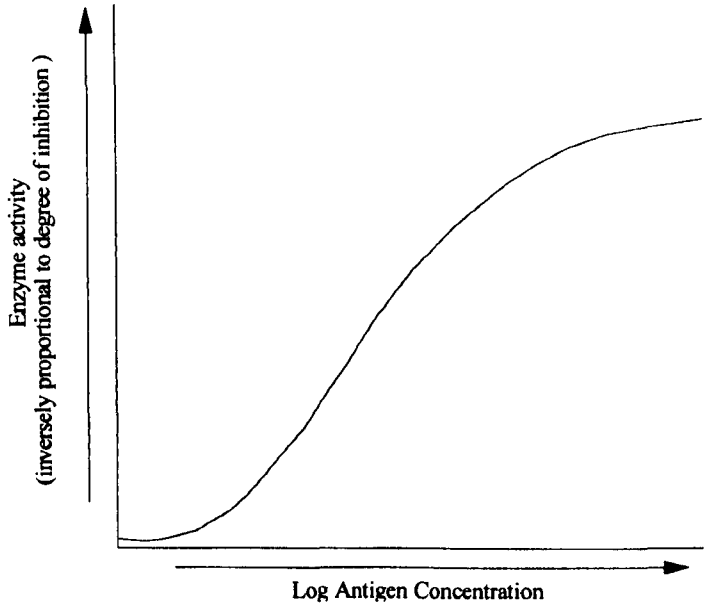


Figure 3.6 Typical semi-logarithmic dose response curve for an EMIT assay. In this technique a known amount of an antigen-enzyme conjugate is added to the sample in the presence of a limited amount of antibody, therefore there is competition for the antibody between the antigen present in the sample and the antigen-enzyme conjugate. If the antigen-enzyme conjugate is bound by the antibody then the enzymic activity is inhibited. The greater the amount of antigen in the sample, the less antigen-enzyme conjugate is bound and therefore there is less inhibition. The result is that the greater the antigen concentration in the sample the greater the enzymic activity.

In assays using colourimetric measurement of enzyme labels, the enzyme action results in a change in absorbance of one of the components of the reaction, similarly fluorimetric assays detect a change in the concentration of a fluorescent component of the reaction. Colourimetric[94,95,274-276] and fluorimetric[277,278] enzyme immunoassays have been developed for the determination of FSH and LH. Likewise, electrochemical enzyme immunoassays have been developed which measure a change in electroactive species within the reaction mixture as a result of enzyme activity. For example, both amperometric[29] and potentiometric[35] enzyme immunoassays have been developed for hCG, which as has already been mentioned, is similarly very similar to LH. Finally, calorimetric assays, that use a thermistor which detects the heat generated by enzymatic reactions,[74,75] have been used for immunological analysis by labelling antibodies with enzymes.[77,78] Luminescence detection of enzyme labels is discussed in section 3.2.7.3

3.2.7.2 Fluorescence Immunoassay

Fluorescent labels can be used for immunoassay, again classification into homogeneous and heterogeneous assays can be made. In homogeneous fluorimmunoassays the properties of the label are modified upon the antibody-antigen reaction taking place. This modification to the signal can be in the form of enhancement, quenching or a change in polarisation.[279] In classical heterogeneous fluorimmunoassays, either antigen or antibody is labelled and assays are performed according to the principles of heterogeneous immunoassays that have already been described. These assays have tended to suffer from problems such as light scattering, quenching and background fluorescence, which has limited their use. However, the use of time-resolved fluorescence techniques has provided methods which are viable alternatives to other detection techniques, and are now commonly used.

Time-resolved fluorescence techniques (e.g. dissociation enhanced lanthanide fluorimetric immunoassay (DELFIA))[280] are based upon the long fluorescent half life of europium chelates. The excitation light is in the form of a flash lasting less than a microsecond, the background fluorescence due to endogenous material in the sample decays rapidly with a half life of less than 50 nanoseconds. Measurement of the fluorescence arising from the europium ion can be measured after hundreds of microseconds. Time-resolved fluorescence techniques for the determination of FSH and LH are commercially available and widely used.[248,260,261,281]

3.2.7.3 Luminescence immunoassay

Luminescence is a term that encompasses a range of processes which produce light. Luminescence, in the forms of chemiluminscence and bioluminescence are increasingly being used to monitor immunological reactions. Chemiluminescence is the emission of light as a result of a chemical reaction, bioluminescence is where the catalytic activity of a protein increases the efficiency of a chemiluminscent process. Luminescence has been used both as a label in its own right, and in reactions involving an enzymatic label. Luminescent reactions are very sensitive and have very low detection limits, as no external light source is required, all the light falling on the detector originates from the luminescent reaction. This means that the background signal is essentially zero, and that detection of a single photon produced in a luminescence reaction is possible.[273] In assays where a luminescent molecule itself is used to label an antibody or antigen, light is generated by the provision of a stimulus to the luminescent molecule, for example in Figure 3.7, the addition of an oxidising agent to luminol results in its conversion to aminopthalate with the production of light. In assays where an enzyme is used as the label, there is a catalytic conversion of the solution phase luminescent molecule For example in Figure 3.7 horseradish peroxidase can

catalyse the conversion of luminol to aminopthalate. Commercial assays based on the measurement of luminescence are available for both FSH and LH.[257,282-285]

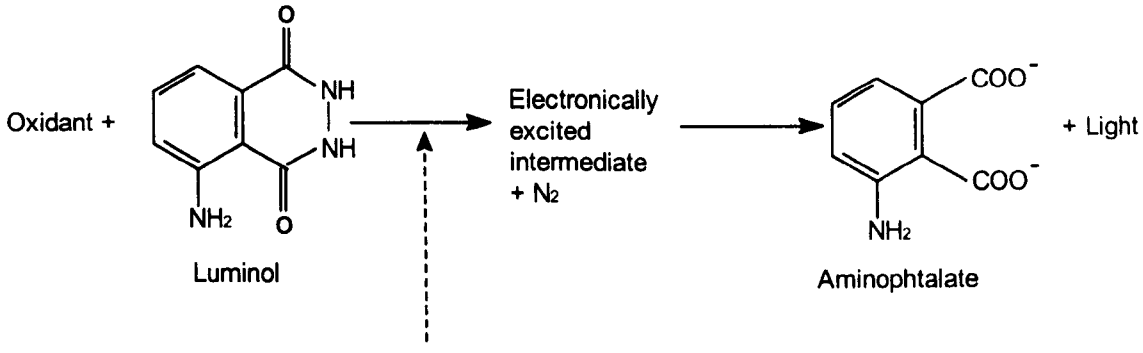


Figure 3.7 Scheme for the chemiluminescent oxidation of luminol.

3.2.8 Near-patient tests

Rapid near-patient tests for the assay of LH have been developed, and are commercially available.[248] These tests are designed to determine when ovulation has occurred by monitoring the LH surge. This information can be used to increase the chance of pregnancy by predicting when a woman is most likely to conceive, but perhaps the most useful application of rapid gonadotrophin measurements is in assisted conception. The availability of a simple and rapid assay makes possible successful and precise timing of ovulation during artificial insemination or ovum retrieval for *in-vitro* fertilisation (IVF) procedures in treatment for infertility.[286] One such near-patient device is the Ovukit™ (formerly known as the Ovutest™), which is a semi-quantitative assay system based upon immunochromatography (the basis of the majority of pregnancy tests). In the Ovukit™ an

antibody specific for LH is immobilised as a line on a membrane. The sample (urine) is applied to one end of the membrane, and "wicks" along the length of the membrane and any LH in the sample is "captured" by the immobilised antibody. A similar device is the Unipath ClearPlan One Step ™ device, this easy to use device can provide a result in just 3 minutes. Visualisation is by means of a conjugate which produces a coloured line visible to the naked eye, if the levels of LH are above a threshold level.[287,288] The assay is optimised so that a positive result is only obtained during the LH surge, and therefore indicates that ovulation is occurring.

3.3 Amperometry

The immunosensor constructed in this work was an amperometric sensor, in which an electrical potential is applied to a sensor electrode, which facilitates reduction or oxidation of electroactive species at the electrode-solution interface. This "redox" reaction involves the transfer of electrons between the electrode and the electroactive species, with a current being produced which is proportional to the concentration of the electrochemical species at the electrode surface. As has already been described, amperometric transducers have been very widely used in biosensing, particularly systems based upon oxygen or hydrogen peroxide electrodes, where the current produced is directly proportional to the amount of oxygen or hydrogen peroxide reduced or oxidised at the electrode. This approach has high sensitivity and a linear concentration dependence over a given concentration range, and improved selectivity over interferents can be obtained by careful choice of the electrode potential. Many molecules (such as proteins) are not intrinsically electroactive, and therefore it is frequently necessary to introduce enzymic labels which can catalyse redox reactions and produce electroactive species. Amperometric sensing of immunological

reactions can be performed by using an immobilised antibody to bind the analyte at the electrode surface, and then adding a second enzyme labelled antibody which is also directed against the analyte.

In amperometric devices the basic requirement is to be able to maintain a working electrode at a constant potential and measure any resultant current. This can best be achieved using a three electrode system, with a working electrode being maintained at a constant potential with respect to a reference electrode, whilst allowing current to flow between the working electrode and a counter electrode in solution. When the size of the current is small ($< \mu A$ cm^{-2}), the configuration can be simplified by removing the counter electrode, and allow the (measured) current to return via a combined reference/counter electrode.

The observed current is a combination of capacitive or "non-Faradaic" currents (which are due to the physical rearrangements of ions in the double layer) and the Faradaic current (which is due to redox reactions). The Faradaic current is a direct measure of the rate of the electrochemical reaction taking place at the electrode. In practice this will depend upon two dominating factors; the rate at which the material arrives at the electrode surface from the bulk solution (mass transport) and the rate at which electrons transfer between the electrode and the electroactive species (charge transfer), which is described by the Butler Volmer equation,[289] and relates to the extent of electron transfer to the applied overpotential and the transfer coefficient.

Mass transport comprises of three components; diffusion, migration and convection. Diffusion is the dispersion of a species within another species, tending to a homogeneous mixture without chemical combination (i.e. movement "down" a concentration gradient).

Migration is the movement of charged species in an electric field, and is therefore dependent upon the magnitude of the field and the charge on the ion. Convection is the movement of analyte molecules due to inducing the physical movement of the bulk solution (e.g. stirring). These three components of mass transport to the electrode are described (in one dimension) by the Nernst-Planck equation.[289] If the migrational and convectional contributions to the mass transport flux can be eliminated then the steady state diffusion limited current can be determined as in equation 3.1. For the elimination of convective transport, all that is required is to keep the solution quiescent, whilst the addition of a supporting electrolyte at a concentration 100 times that of the electroactive species, essentially eliminates migration by dissipating the electric field over all of the ions in solution, thus "shielding" those ions of interest from electro-migrational forces).

$$i(t) = nFAD\frac{\delta C(x,t)}{\delta x}\int_{x=0} \qquad (3.1)$$

where n is the number of electrons involved in the reaction (Faraday equivalents mol^{-1}), A is the electrode area, (cm^2), D is the diffusion coefficient ($cm^2\ s^{-1}$), C is the concentration (mol cm^{-3}) and F= 96,484.6 C $Faraday^{-1}$.

3.3.1 Chronoamperometry

Chronoamperometry is a technique where an applied potential is stepped from an initial value to a different value at which the potential is held for a period of time (i.e. a square wave). The resultant current is recorded as a function of time. A typical chronoamperometric response curve displays a sharp current spike (at the time at which the potential step occurs and consisting of both Faradaic and non-Faradaic components) which

decays with time. In chronoamperometry, the applied potential is sufficient to reduce the electrode surface concentration of the electroactive species essentially to zero.[289] The effect of the capacitive current becomes less evident with time and the Faradaic current (which is a direct measure of the rate of the electrochemical reaction taking place at the electrode) dominates. Typical chronoamperometric input and output waveforms are shown in Figure 3.8

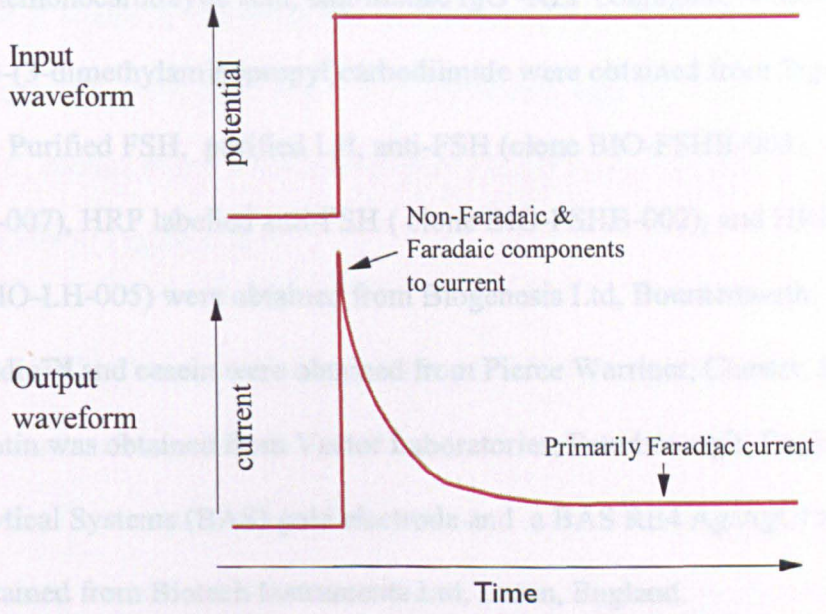


Figure 3.8 Typical input and ouput chronoamaperometry waveforms. The input waveform comprises of an applied potential which is stepped from an initial value to a different value at which the potential is held for a period of time (i.e. a square wave). The resultant current is recorded as a function of time (output waveform). A typical chronoamperometric response curve displays a sharp current spike (at the time at which the potential step occurs and consisting of both Faradaic and non-Faradaic components) which decays with time. In chronoamperometry the applied potential is sufficient to reduce the electrode surface concentration of the electroactive species essentially to zero. The effect of the capacitive current becomes less evident with time and the Faradaic current (which is a direct measure of the rate of the electrochemical reaction taking place at the electrode) dominates.

3.4 Materials

Positive S1400-31 photoresist and Microposit developer were obtained from Shipley Europe, Coventry, England. Silicon wafers were obtained from Shin Etsu, Livingston, Scotland; NiChrome (80:20 Ni:Cr) and gold were obtained from Goodfellow Cambridge Ltd, Cambridge, England.

Ferrocenemonocarboxylic acid, anti-mouse IgG -ALP conjugate, N-acetyl-l-cysteine, and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide were obtained from Sigma, Poole, England. Purified FSH, purified LH, anti-FSH (clone BIO-FSHB-003), anti-LH (clone BIO-LH-007), HRP labelled anti-FSH (clone BIO-FSHB-002), and HRP labelled anti-LH (clone BIO-LH-005) were obtained from Biogenesis Ltd, Bournemouth, England. Neutravidin™ and casein were obtained from Pierce Warriner, Chester, England. Photobiotin was obtained from Vector Laboratories, Peterborough, England. A Bioanalytical Systems (BAS) gold electrode and a BAS RE4 Ag/AgCl reference electrode were obtained from Biotech Instruments Ltd, Luton, England.

Instrumentation used comprised a EG&G 273A potentiostat (EG & G, Sunninghill, England), Bioanalytical System CV-37 potentiostats (Biotech Instruments Ltd, Luton, England), a Goerz SE120 dual channel chart recorder (Belmont Instruments, Glasgow, UK), a 100W HB-10101AF super high pressure mercury vapour lamp (Nikon, Tokyo, Japan) and a photolithographic mask aligner (HTG San Jose, California, USA).

3.5 Method

3.5.1 Photolithographic Lift Off patterning techniques for the fabrication of electrodes

Positive organic resists such as Shipley S1400-31 photoresist are composed of long molecular chains with high molecular weight. Upon exposure to ultraviolet (UV) light, extensive chain scission takes place resulting in fragmented molecules with low molecular weight. A developer which dissolves the molecules at a rate dependent upon their molecular weight is then employed. Those regions that have been exposed to ultraviolet light, with molecules of relatively low molecular weight dissolve rapidly. Unexposed high molecular weight regions dissolve relatively slowly. Therefore, after development, the resist image has "windows" opened where it has been exposed to UV light. This process is shown schematically in Figure 3.9a-d.

In the "lift-off" technique, metal is evaporated from a point source and is deposited both onto the resist and onto the substrate through the resist windows. The resist is then dissolved by immersion in a resist solvent, causing the overlying metallisation to lift off, therefore only the metal that has been deposited directly onto the substrate through the resist window remains. This process is shown schematically in Figure 3.9e-f.

Areas of the deposited metal can subsequently be electrically insulated from the environment by patterning a second layer of photoresist on to the surface. On this occasion only the areas of the metallised surface that one wishes exposed to the environment are irradiated, after removing the resist from these areas the remaining resist is hardened in an oven and can then act as an electrical insulator This process is shown schematically in Figure 3.9g-h.

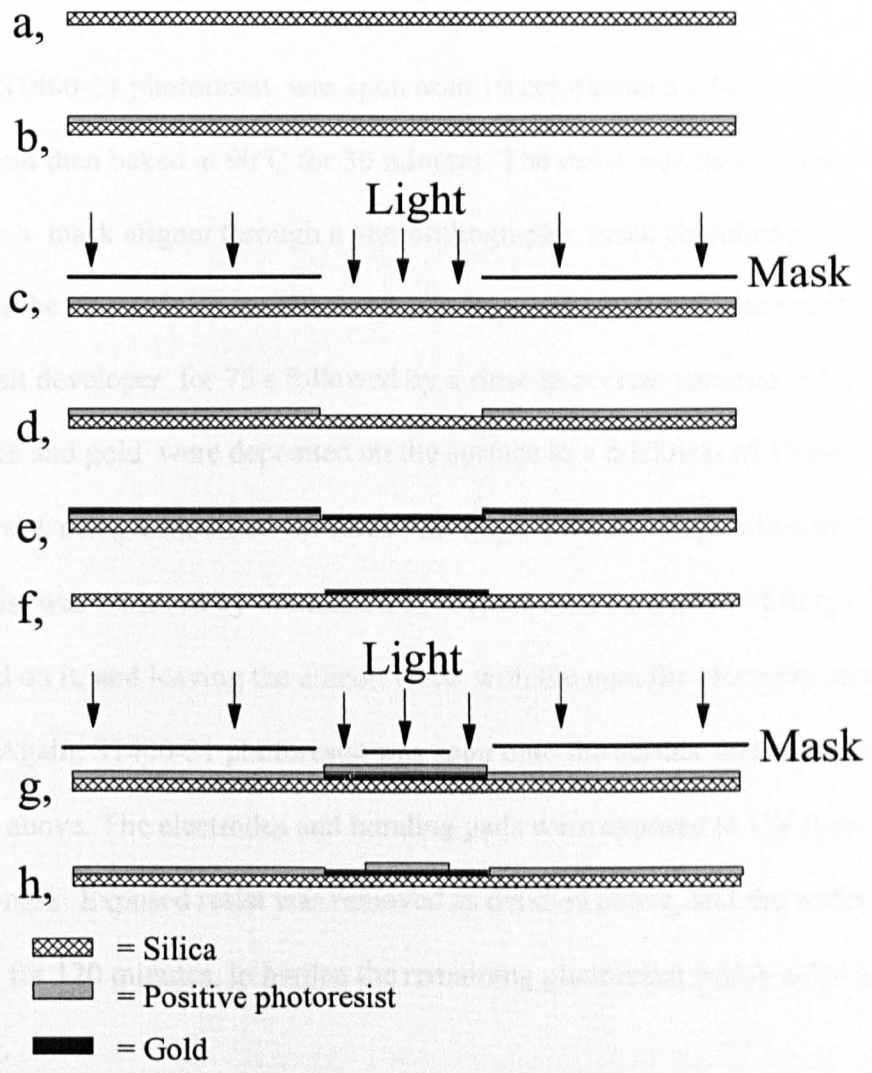


Figure 3.9 Fabrication of electrodes using photolithographic lift-off techniques. A substrate (typically a silicon wafer) is cleaned (a) and coated with positive photoresist (b). The surface is exposed to UV light though a mask (c). A developer dissolves the resist that has been exposed to ultraviolet light (d). Metal is evaporated from a point source and is deposited both onto the entire surface (e). The resist is then dissolved by immersion in a resist solvent, causing the overlying metallisation to lift off, therefore only the metal that has been deposited directly onto the substrate through the resist window remains (f). Areas of the deposited metal can be electrically insulated by patterning a second layer of photoresist on to the surface. Only the areas of the metallised surface that one wishes exposed to the environment are irradiated (f), after removing the resist from these areas the remaining resist is hardened in an oven and can then act as an electrical insulator (h)

3.5.2 Electrode fabrication

Positive S1400-31 photoresist was spun onto 10 cm diameter silicon wafers at 4000 rpm for 30 s and then baked at 90°C for 30 minutes. The resist was then illuminated by light for 12 s from a mask aligner through a photolithographic mask containing dark field relief images of the electrode arrays (Figure 3.10). Exposed resist was removed by immersion in Microposit developer for 75 s followed by a rinse in reverse osmosis (RO) water. NiChrome and gold were deposited on the surface to a thickness of 10nm and 100nm respectively, using a modified Edwards 12E High Vacuum evaporation unit. The remaining photoresist was removed by immersion in acetone for 20 minutes, "lifting off" the metal deposited on it, and leaving the silicon wafer with the metallic electrode arrays on its surface. Again, S1400-31 photoresist was spun onto the surface and baked at 90°C as detailed above. The electrodes and bonding pads were exposed to UV light through a second mask. Exposed resist was removed as detailed above, and the wafer was then baked at 120°C for 120 minutes, to harden the remaining photoresist which acted as an electrical insulator.

Ag/AgCl reference electrodes were prepared by electrosorbtion of silver onto specified gold electrodes from a solution of 0.1M $AgNO_3$ in 0.1M sulphuric acid with a silver anode (at a constant current of 0.4 mA cm^{-2} for 6 hours), followed by chloridisation in 0.1M HCl (0.4 mA cm^{-2} for 30 minutes).

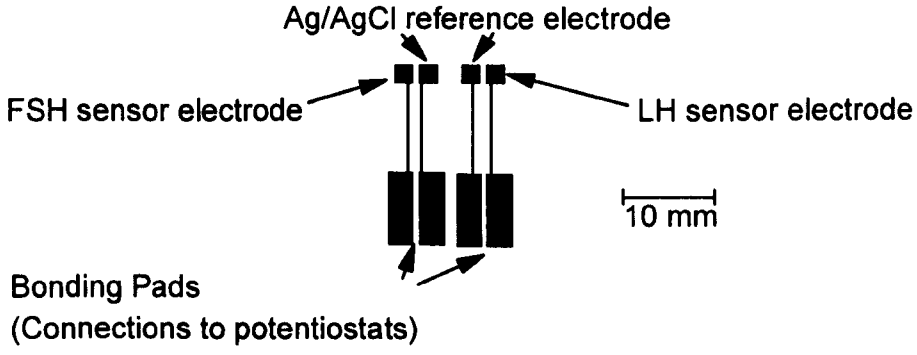


Figure 3.10 Diagrammatic representation of the fabricated electrode array for use in the construction of a multianalyte immunosensor.

The electrochemical behaviour of the fabricated electrodes was verified by comparison of cyclic voltammograms (-0.2 to +0.75 V, 20 mV s^{-1}) of 0.2 mM ferrocenemonocarboxylic acid in 50 mM Tris 50 mM KCl, pH 7.4 with those obtained using a BAS gold working electrode and a BAS RE4 Ag/AgCl reference electrode. Reproducibility of the fabricated electrode arrays, was assessed by measuring the chronoamperometric response (10 s at 0V, 30 s at +650 mv)) to 0.5 mM H_2O_2 in 50 mM sodium phosphate buffer containing 50 mM KCl, pH 7.4. These studies were performed using an EG&G 273A potentiostat.

3.5.3 Antibody immobilisation

Electrode arrays were incubated in 2 mM NAC in 10 mM phosphate buffer (pH 7.0) for 120 minutes at room temperature, followed by 120 minutes incubation in 1% (w/v) EDC in 10 mM phosphate buffer (pH 7.0). Note that the use of NAC provides an "open" self assembled monolayer (in contrast to highly organised SAMs), and this does not "block" the gold electrochemical surface. The electrode arrays were then incubated in 100μg ml^{-1} Neutravidin in 10 mM phosphate buffer (pH 7.0) for 16 hours at 4°C. All subsequent stages of the immobilisation procedure were performed at room temperature. After washing in PBS, the electrodes were first incubated in 10 mg ml^{-1} casein in PBS for 60 minutes and then in 10 μg ml^{-1} long arm photobiotin in PBS, for 20 minutes in the dark. All subsequent stages of the immobilisation procedure were performed in the dark. After washing in PBS the wafer was covered with 10 μg ml^{-1} monoclonal anti-FSH (clone BIO-FSHB-003), and selected electrodes were exposed to light from a mercury vapour lamp, using a suitable mask as descibed in section 2.3.1. After washing in PBS, the wafer was covered with 10 μg ml^{-1} monoclonal anti-LH ((Biogenesis clone LH-007), and selected electrodes were exposed to light from the lamp for 15 minutes. After washing in PBS, the entire wafer was exposed to light from the lamp for 15 minutes in the presence of 10 mg ml^{-1} casein in PBS, and again washed in PBS.

3.5.4 Immunoassay procedure

The immunoassay procedure used was a "sandwich" ELISA technique, with the immobilised "capture" antibodies on the electrode surfaces, and the subsequent addition of enzyme labelled antibodies directed against second epitopic sites on the antigens (Figure 3.11). Sensors were incubated with 250 µl of sample for 60 minutes, washed thoroughly with PBS, and incubated in a mixture of 10 µg ml^{-1} horseradish peroxidase (HRP) labelled anti-FSH ((Biogenesis clone BIO-FSHB-002), and 10 µg ml^{-1}HRP labelled anti-LH((Biogenesis clone LH-005) in PBS, for 60 minutes at room temperature, followed by further washing in PBS.

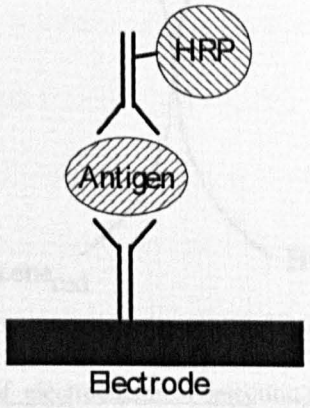


Figure 3.11 Principle of the "sandwich" ELISA used in the determination of LH and FSH. The specific capture antibody (BIO-FSHB-003 for FSH and BIO-LH-007 for LH) was immobilised onto the electrode surface using the avidin - photobiotin patterning technique. Upon exposure to the sample the antibody bound its complimentary antigen, and unbound material was washed away. A second specific antibody (BIO-FSHB-002 for FSH and BIO-LH-005 for LH) conjugated to HRP was added, and this bound to a second discrete epitope on the antigen. Therefore, the amount of HRP attached to the electrode surface was proportional to the concentration of the antigen in the sample.

Simultaneous assessment of HRP activity at the two sensors was performed chronoamperometrically using two Bioanalytical System CV-37 potentiostats and a Goerz SE120 dual channel chart recorder. Activity was determined by measuring the current produced at 20 s in the presence of 10 mM hydrogen peroxide, and 0.2 mM ferrocenemonocarboxylic acid in 50 mM phosphate buffer containing 50 mM KCl, pH 7.4, with a potential of +150 mV applied to the working electrode versus the Ag/AgCl reference electrode.[290] The principle of using ferrocenemonocarboxylic acid as an electron mediator is illustrated schematically in Figure 3.12

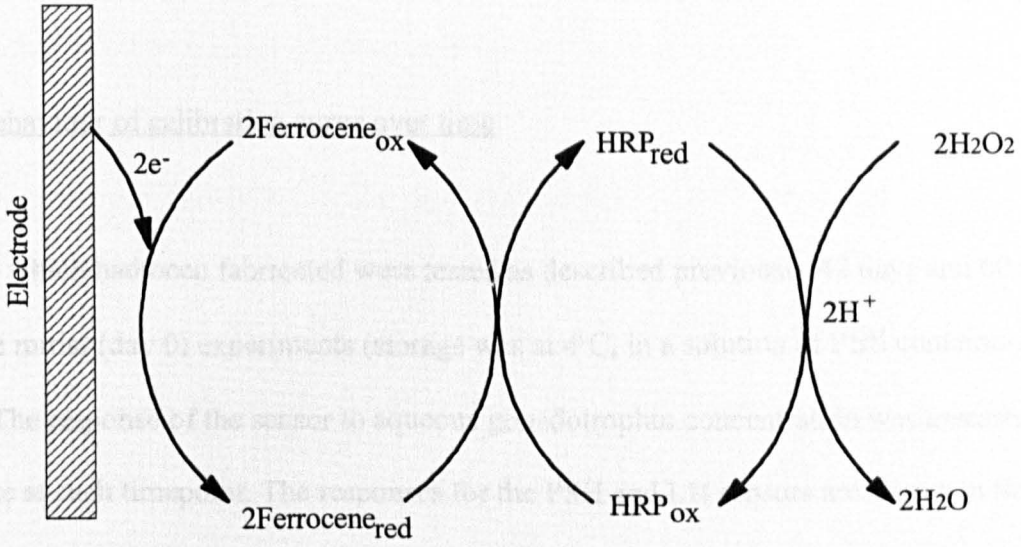


Figure 3.12 Schematic representation of electrochemical detection of HRP using ferrocene derivatives. As HRP catalyses the conversion of H_2O_2 to H_2O, it is oxidised in the process. HRP is reconverted into the reduced form by the ferrocene (bis(h^5-cyclopentadienyl)iron) derivative (in this case ferrocenemonocarboxylic acid), which is itself oxidised in the process and is subsequently reduced by gaining electrons from the electrode, resulting in current flow.

The response of the immunosensors to hormone concentration in a buffered aqueous solution was determined by preparation of a series of standards (0 - 100 U l^{-1}) of FSH and LH (Biogenesis) which covered the concentration range of clinical interest. The results obtained (Section 3.6.2) were subsequently used as calibration curves for further studies. The multianalyte immunosensor was used to determine gonadotrophin concentrations in 10 serum samples from hospital outpatients (with a representative range of values typically seen in clinical laboratories), and the results were compared with those obtained using an established DELFIA method.[291] Assays using the immunosensor were performed in triplicate, and the results obtained are shown in Section 3.6.2.

3.5.5 Behaviour of calibration curve over time

Sensors which had been fabricated were tested as described previously 42 days and 60 days after the initial (day 0) experiments (storage was at 4°C, in a solution of PBS containing 1% BSA). The response of the sensor to aqueous gonadotrophin concentration was assessed in triplicate at each timepoint. The responses for the FSH and LH sensors are shown in Section 3.6.3. In order to check that the immobilised antibody was not desorbing from the surface upon storage, the sensors were also incubated with an anti-mouse IgG - ALP conjugate at each time point. Following washing in TBS and incubation in the conjugate for 60 minutes, the sensors were washed extensively in TBS and their ALP activity was determined colourimetrically using pNPP as the substrate as described in Section 2.5.2.1. The absorbance was measured at 404 nm, and compared with a that of a freshly prepared reference solution (1:60000 dilution of stock conjugate), to take account of inter-assay variation.

3.6 Results

3.6.1 Electrochemical Characterisation of Electrodes

The cyclic voltammograms of 0.2 mM ferrocenemonocarboxylic acid in 50 mM Tris 50 mM KCl, pH 7.4 (-0.2 to +0.75 , 20 mV s^{-1}) for the fabricated electrodes and for commercial (BAS) electrodes are shown in Figure 3.13. The potentials at which oxidation and reduction peaks were evident upon cyclic voltammetry for the fabricated electrodes were within 5 mV of those obtained when using the BAS electrodes (E_{pa} = 0.355V, E_{pc} = 0.296V), demonstrating that the fabricated electrode arrays behaved similarly to a combination of commercially obtained gold and Ag/AgCl electrodes.

The intra-batch coefficient of variation for the responses of the electrode arrays to 0.5 mM H_2O_2 in 50 mM sodium phosphate buffer containing 50 mM KCl, pH 7.4 (assessed by measuring the chronoamperometric response (10 s at 0V, 30s at +650 mV vs. Ag/AgCl, current measured 30 s after application of +650mV potential)) was 1.86% (n=20), whilst the inter-batch coefficient of variation was 2.43% (n=5), thus demonstrating that the response (and therefore the manufacture) of the fabricated electrode arrays were both reproducible.

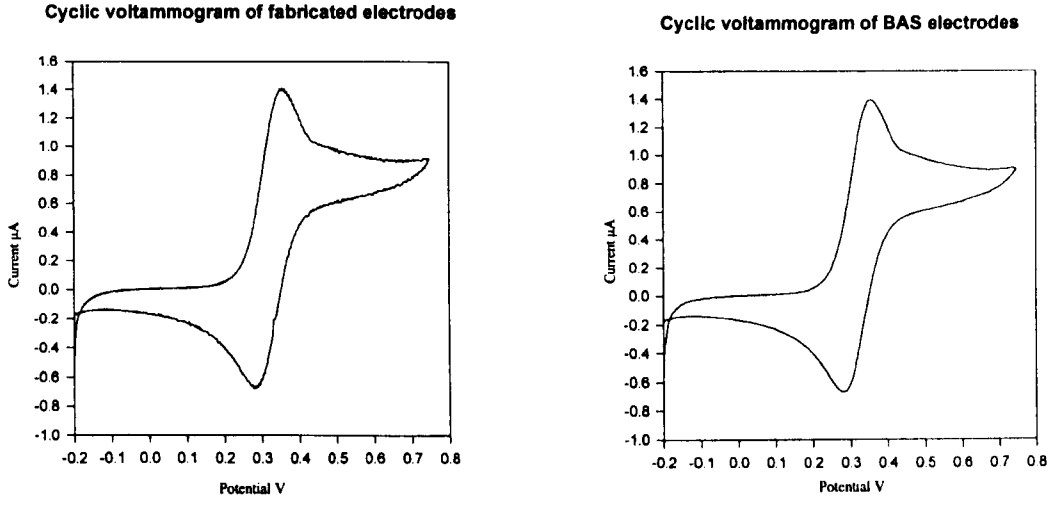


Figure 3.13 Cyclic voltammograms of ferrocenemonocarboxylic acid obtained using fabricated electrode pair and Commercial (BAS) electrodes The electrochemical behaviour of the fabricated electrodes (manufacture described in 3.5.1) was verified by comparison of cyclic voltammograms (-0.2 to +0.75 V vs. Ag/AgCl, 20 mV s^{-1}) of 0.2 mM ferrocenemonocarboxylic acid in 50 mM Tris 50 mM KCl, pH 7.4 with those obtained using a BAS gold working electrode and a BAS RE4 Ag/AgCl reference electrode. The potentials at which oxidation and reduction peaks were evident upon cyclic voltammetry of ferrocene- monocarboxylic acid for the fabricated electrodes were within 5 mV of those obtained when using the BAS electrodes (E_{pa} = 0.355V, E_{pc} = 0.296V)

3.6.2 Response of immunosensors to aqueous and clinical samples

The response of the sensor to FSH and LH in buffer is shown in Figures 3.14 and 3.15, the current (a result of the enzymic activity) is clearly linearly related to the antigen concentration over the range 0 to 100 U l^{-1}. When corrected for the specific activities of the hormone preparations these ranges equate to 0 to 26 ng l^{-1} and 0 to 18 ng l^{-1} for FSH and LH respectively. Figure 3.14 demonstrates that the current produced by the FSH sensor is proportional to the FSH concentration, and that cross reactivity with LH is negligible. The response to FSH is 2.11 nA / U l^{-1} (8.018 nA / ng l^{-1}) whilst the response to LH is 0.07 nA / U l^{-1} (0.385 nA / ng l^{-1}).

Data shown in Figure 3.15 demonstrates that the current produced by the LH sensor is proportional to the LH concentration, and that cross reactivity with FSH is negligible. The response to LH is 2.48 nA / U l^{-1} (13.64 nA / ng l^{-1}) whilst the response to FSH is 0.11 nA / U l^{-1} (0.418 nA / ng l^{-1}).

Figures 3.16 and 3.17 show comparisons of results obtained for human serum samples from the multianalyte sensor, with those from an established DELFIA technique. There is a very good correlation between methods, and close agreement between results at all concentrations for both FSH ($r = 0.9852$) and, LH ($r = 0.9783$).

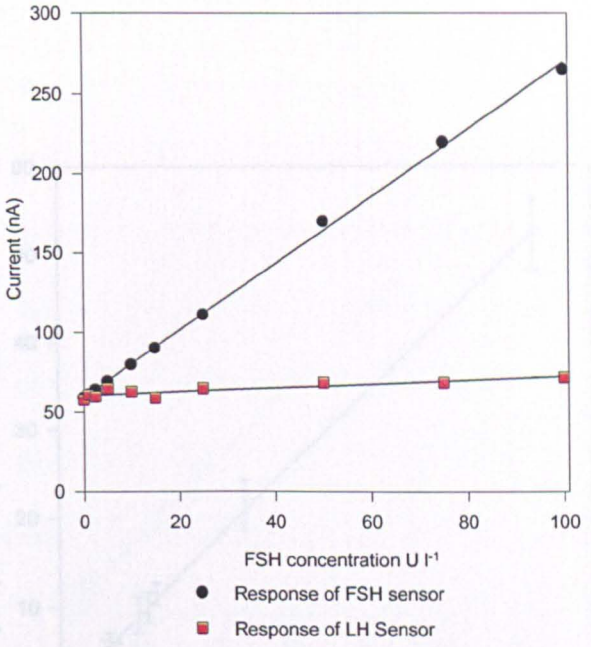


Figure 3.14 Response of multianalyte sensor to FSH concentration in buffer The response of the immunosensors to FSH concentration in a buffered aqueous solution was determined by preparation of a series of standards (0 - 100 U l^{-1}) of FSH (LH concentration = 0). Monoclonal anti-FSH and monoclonal anti-LH antibodies were immobilised on selected sensors using the avidin-photobiotin patterning technique.

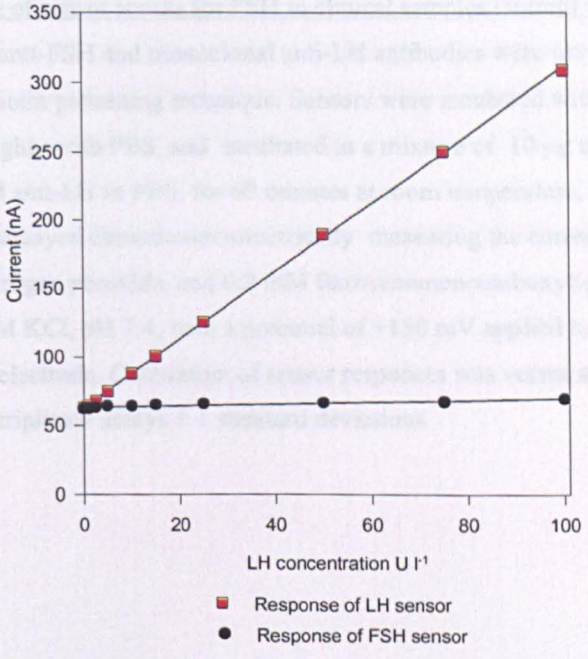


Figure 3.15 Response of multianalyte sensor to LH concentration in buffer. The response of the immunosensors to LH concentration in a buffered aqueous solution was determined by preparation of a series of standards (0 - 100 U l^{-1}) of LH (FSH concentration = 0). Monoclonal anti-FSH and monoclonal anti-LH antibodies were immobilised on selected sensors using the avidin-photobiotin patterning technique.

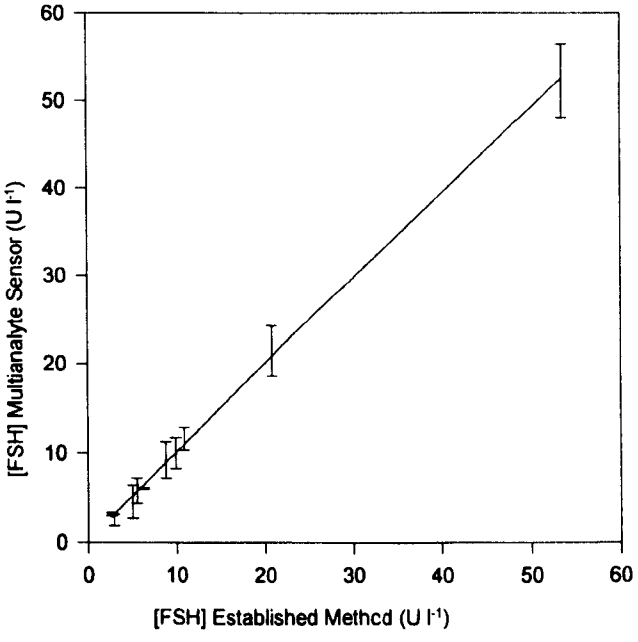


Figure 3.16 Comparison of sensor results for FSH in clinical samples (serum) with an established DELFIA technique. Monoclonal anti-FSH and monoclonal anti-LH antibodies were immobilised on selected sensors using the avidin-photobiotin patterning technique. Sensors were incubated with 250 µl of sample for 60 minutes, washed thoroughly with PBS, and incubated in a mixture of 10 µg ml^{-1} HRP labelled anti-FSH and 10 µg ml^{-1} HRP labelled anti-LH in PBS, for 60 minutes at room temperature, followed by further washing in PBS. HRP activity was assayed chronoamperometrically measuring the current produced at 20 s in the presence of 10 mM hydrogen peroxide, and 0.2 mM ferrocenemonocarboxylic acid in 50 mM phosphate buffer containing 50 mM KCl, pH 7.4, with a potential of +150 mV applied to the working electrode versus the Ag/AgCl reference electrode. Calibration of sensor responses was versus aqueous solutions of FSH. Error bars represent mean of triplicate assays ± 1 standard deviations

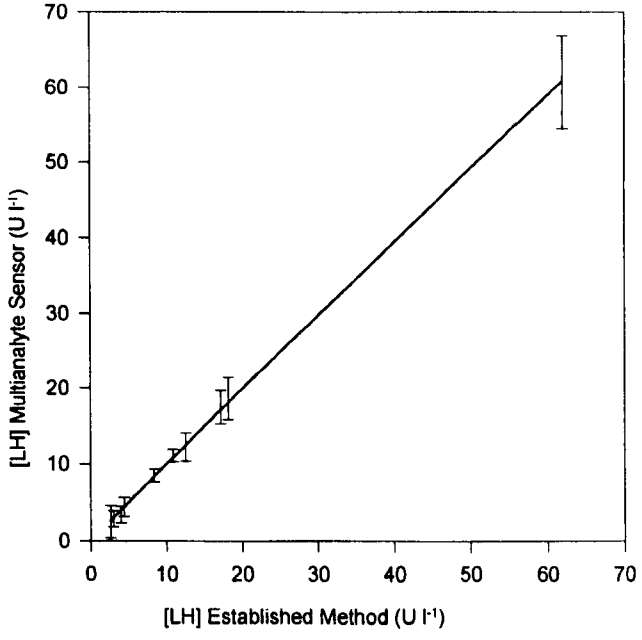


Figure 3.17 Comparison of sensor results for LH in clinical samples (serum) with an established DELFIA technique. Monoclonal anti-FSH and monoclonal anti-LH antibodies were immobilised on selected sensors using the avidin-photobiotin patterning technique. Sensors were incubated with 250 µl of sample for 60 minutes, washed thoroughly with PBS, and incubated in a mixture of 10 µg ml^{-1} HRP labelled anti-FSH and 10 µg ml^{-1} HRP labelled anti-LH in PBS, for 60 minutes at room temperature, followed by further washing in PBS. HRP activity was assayed chronoamperometrically measuring the current produced at 20 s in the presence of 10 mM hydrogen peroxide, and 0.2 mM ferrocenemonocarboxylic acid in 50 mM phosphate buffer containing 50 mM KCl, pH 7.4, with a potential of +150 mV applied to the working electrode versus the Ag/AgCl reference electrode. Calibration of sensor responses was versus aqueous solutions of LH. Error bars represent mean of triplicate assays ± 1 standard deviations.

3.6.3 Behaviour of calibration curve over time

The responses for sensors stored at 4°C for 0, 42 and 60 days to aqueous FSH and LH are shown in Figures 3.18 and 3.19 respectively. It can be seen from these figures that there was a significant decrease in the response (in terms of current) of both sensors upon storage. After 42 days storage at 4°C, the FSH sensor's response was 79% of that after preparation (Day 0) (coefficient of variation (CV)=8.2%, n=3), whilst after 60 days the response was 70% (CV=11.1%, n=3) of the initial value. After 42 days storage at 4°C, the LH sensor's response was 69% of that at Day 0 (CV=9.7%, n=3), whilst after 60 days the response was 62% (CV=6.6%, n=3) of the initial value.

The amount of antibody immobilised on the sensor surfaces as demonstrated by testing with an anti-mouse IgG-ALP conjugate did not show any variation with time (Day 0 sample:reference ratio = 2.17 (CV=7.8 , n=3), Day 42 sample:reference ratio = 2.04 (CV=9.2 , n=3), Day 60 sample:reference ratio = 2.22 (CV=6.2 , n=3)).

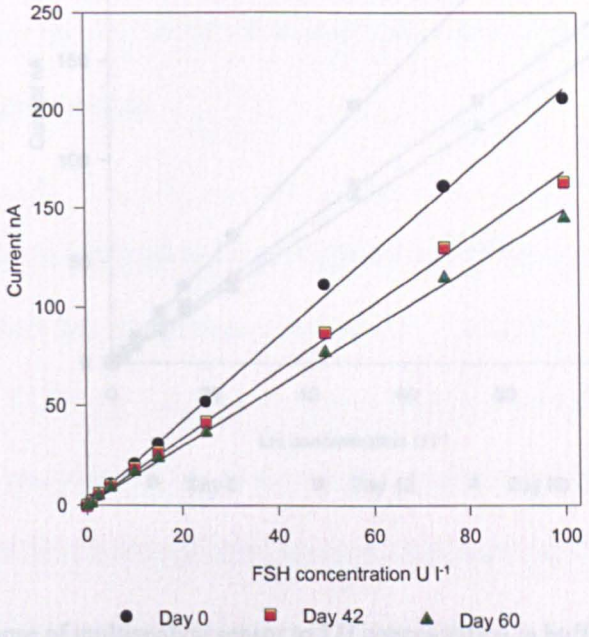


Figure 3.18 Response of multianalyte sensor to FSH concentration in buffer over a 60 day period. The response of the immunosensors to FSH concentration in a buffered aqueous solution was determined by preparation of a series of standards (0 - 100 U l^{-1}) of FSH (LH concentration = 0). Monoclonal anti-FSH and monoclonal anti-LH antibodies were immobilised on selected sensors using the avidin-photobiotin patterning technique. Immunoassay with chronoamperometric detection was performed as described in Section 3.5.4 . The response of the sensors was measured on the day of manufacture and after storage at 4°C for 42 and 60 days. Each point on the plot is the mean response obtained from performing the assay using three sensors.

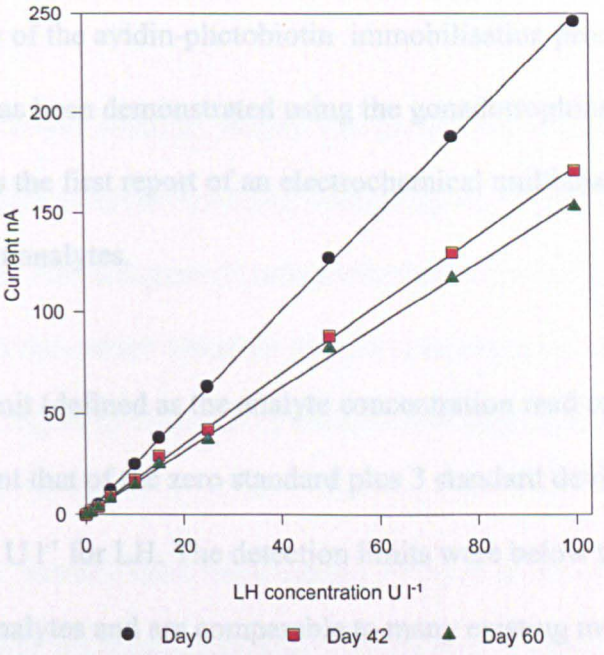


Figure 3.19 Response of multianalyte sensor to LH concentration in buffer over a 60 day period.

The response of the immunosensors to LH concentration in a buffered aqueous solution was determined by preparation of a series of standards (0 - 100 U l^{-1}) of LH (FSH concentration = 0). Monoclonal anti-FSH and monoclonal anti-LH antibodies were immobilised on selected sensors using the avidin-photobiotin patterning technique. Immunoassay with chronoamperometric detection was performed as described in Section 3.5.4 . The response of the sensors was measured on the day of manufacture and after storage at 4°C for 42 and 60 days. Each point on the plot is the mean response obtained from performing the assay using three sensors.

3.7 Discussion

The applicability of the avidin-photobiotin immobilisation procedure to multianalyte immunoassays has been demonstrated using the gonadotrophins FSH and LH as a model system.[292] This is the first report of an electrochemical multianalyte immunosensor for high molecular weight analytes.

The detection limit (defined as the analyte concentration read off the standard curve, for a current equivalent that of the zero standard plus 3 standard deviations (n=10)) was 2.1 U l^{-1} for FSH and 1.8 U l^{-1} for LH. The detection limits were below the bottom of the reference range for both analytes and are comparable to many existing methods,[261] although lower detection limits have been reported in some other methods.[278] It is likely that further optimisation of the reagents and assay protocol used in the immunosensor assay would result in a lower detection limit. Over the working range of the assays (0 to 100 U l^{-1}), the concentration:current relationship is linear for both analytes, and the immunosensor showed excellent correlation with an established analytical technique upon testing clinical samples. The replicate (×3) analysis of the 10 patient samples shows acceptable imprecision (R.M.S. CV% = 5.2% for FSH and 6.7% for LH).

The variation in the current recorded for a given concentration of hormone on different dates could be due to a number of factors. Minor variations in analytical conditions may have been a factor (e.g. changes in room temperature), but as there appeared to be a progressive decrease in current obtained for a given concentration with time, it is likely that there was a loss of biological activity of the antibodies and / or the enzyme label. The lack

of variation in the quantity of antibody on the sensor with time (as demonstrated with the use of anti mouse IgG-ALP conjugate) indicates that the decreased response was not due to desorption of the capture antibody from the sensor surface.

The current that flowed when no antigen was present was due to a number of factors, chief amongst these was the current resulting from electrochemical processes unrelated to the immunoassay (i.e. the background current obtained when there was no enzymic activity). The remainder of the current when the antigen concentration was zero was due to either to non-specific binding or to diffusion of electroactive species between electrodes.

As discussed in Chapter 2 there are a number of types of non-specific binding in solid-phase enzyme immunoassays. One of these occurs where an inappropriate antibody binds at a sensor site (e.g. anti-LH on a sensor for FSH), a circumstance which can come about for a number of reasons, such as binding through non-specific protein-protein interactions, hydrophobic interactions with non-polar surfaces, or electrostatic interactions between the protein and the surface.[124-126] This inappropriate antibody is subsequently able to bind it's complimentary antigen and the enzyme labelled second antibody. Sample components themselves can bind non-specifically to sensors, by the same processes as described above (e.g. LH reacting non-specifically with anti-FSH). Subsequently, if enzyme labelled-antibody to this sample component is added a "false" signal can be produced. The labelled antibodies themselves can also bind non-specifically to the sensors with the same effect. Antibody "cross-reactivity" can also cause spurious results, this can be minimised by the use, as in this study, of highly specific monoclonal antibodies. As demonstrated in Chapter 2, the use of deglycosylated avidin (e.g. Neutravidin or streptavidin) to precoat the electrodes, decreases the amount of non-specific binding, particularly that due to protein

interactions with the surface. This enabled the multianalyte immunosensor to be fabricated, with serial immobilisation of sensor antibodies. Casein was used to block unreacted photobiotin groups and to further decrease the effect of non-specific binding.

It is likely that the non-specific binding observed in this study comprised of several or all of the above types. The binding of a labelled antibody to the sensor that is not mediated by an antigen, is independent of sample composition, whereas "antigen mediated" non-specific binding will increase with the increasing concentration of the antigen in the sample. In this study there was a slight increase in the non-specific signal with increasing antigen concentration for both sensors, possibly indicating that some LH was being bound at the FSH sensor and *vice versa.* Alternatively this increase in non-specific signal could be due to the diffusion of increased concentrations of electroactive species between electrodes, although work presented in Chapter 4 would suggest that this is unlikely.

Simultaneous multianalyte immunoassays, where several analytes are measured by a simple procedure, are required in a number of clinical situations, such as the measurement of hormones related with thyroid function and the measurement of gonadotrophins for the investigation of infertility etc. Particular examples where the use of immunosensors for the measurement of gonadotrophins are likely to be beneficial, are as an aid to conception either "naturally" by determining when a woman is most likely to conceive, or for *in vitro* fertilisation procedures. In these cases the rapid measurement of gonadotrophins is extremely important as the rapid transient elevation of gonadotrophins (particularly LH) is an indication of ovulation.[287,288,293] Existing multianalyte immunosensors tend to be qualitative assays, examples of such systems are the Triage™ system for qualitative assays of drugs of abuse and the Spectral Diagnostics assay for markers of myocardial

infarction.[120,121] These systems are based upon immunochromatography assays, which are observed visually and are generally unsuitable for quantitative assay.

Although initially a sensor for measuring two analytes has been constructed, the technology that has been developed is compatible with the fabrication of a sensor for as many analytes as is required.[294] The fabrication and immobilisation procedures used in this work would be suitable for larger scale production using technology commonplace in the microelectronics industry. Additionally, there is no waste of expensive proteins such as monoclonal antibodies as non-immobilised excess protein can easily be recovered, and be reused.

CHAPTER 4

Let us not take it for granted that life

exists more fully in what is commonly thought big

than in what is commonly thought small.

Virginia Woolf

The Common Reader

4 Introduction

Another objective of this work was to investigate the possibility of miniaturising the immunosensor electrodes, so as to develop microspot immunoassays as described by Ekins and co-workers.[295-297] High sensitivity assays based upon the microspot had previously been developed using fluorescence detection, but no there were no reports of the attempted extension of this technology to systems using electrochemical detection. Miniaturisation of electrodes, along with the protein patterning technique already described, would allow the fabrication of arrays of small sensors capable of simultaneously measuring numerous analytes, the so called "immunological compact disc".

Section 4.1 provides a background to antibody antigen reactions at surfaces, Section 4.2 provides a theoretical expansion of previously published work on microspot immunoassays with particular reference to microelectrode arrays. Practical work performed to ascertain the suitability of microspot electrochemical assays is presented in the remaining sections of the chapter.

Practical work was performed to:

1) Ascertain if the formation of hemispherical diffusion fields associated with ultramicroelectrodes caused problems in the use of multianalyte sensors.

2) Ascertain if the diffusion of electrochemical species generated at one electrode to other electrodes caused problems in the use of multianalyte sensors, in particular those employing ultramicroelectrodes.

3) Construct an enzymic biosensor using ultramicroelectrodes capable of simultaneously measuring two analytes.

4) Construct a multianalyte immunosensor using ultramicroelectrodes and capable of simultaneously measuring two analytes.

4.1 Antibody antigen reactions at surfaces

In order to investigate the feasibility of developing an amperometric dual label microspot immunoassay, a degree of theoretical analysis of the principles of immunoassay is required. The equilibrium constant (K) is given by equation 4.1 where $[Ag]$ = concentration of analyte and $[Ab]$ = concentration of the antibody (in M^{-1}).

$$K = \frac{[AbAg]}{[Ab][Ag]} \quad (4.1)$$

All immunoassays are based upon the measurement of the proportion of available antibody binding sites that are bound to the antigen. It can be shown that the fractional occupancy (F) of antibody binding sites by antigen at equilibrium is given by equation 4.2.[297]

$$F^2 - F(\frac{1}{[Ab]} + \frac{[Ag]}{[Ab]} + 1) + \frac{[Ag]}{[Ab]} = 0 \quad (4.2)$$

where $[Ag]$ = concentration of analyte and $[Ab]$ = concentration of the immobilised sensor antibody (both in units of the reciprocal of the equilibrium constant (1/K).

From this equation, it can be shown that as $[Ab]$ tends to 0,

$$F \approx \frac{[Ag]}{[1+Ag]} \quad (4.3)$$

Equation 4.3 implies that the fractional occupancy is dependent on $[Ag]$ and independent of the total amount of immobilised sensor antibody. The effect of this is that when very small amounts of immobilised sensor antibody are used, the assay becomes independent of sample volume. In practice this applies when analyte depletion is less than 1%.[294] Assays

that are independent of sample volume have a number of advantages, particularly in their ease of use if they are to be used in non-laboratory environments.

4.2 microspot immunoassay

If it is assumed that antibody molecules exist with a uniform surface density on a support, then a change in surface area over which the antibody is distributed implies a change in antibody concentration. For example, as shown below if an antibody has an affinity constant (K) of 10^{11} M^{-1}, the total incubation volume is 1 ml and the antibody surface density is 6000 molecules/μm^2, a surface area of 10^5 μm^2 (0.1mm^2) corresponds to an antibody concentration of 0.1K.

6000 molecules/μm^2 $\times 10^5$ $\mu m^2 = 6 \times 10^8$ molecules

6×10^8 molecules $\div$ 6.02×10^{23} molecules $\times$ $1 \times 10^3 \approx 1 \times 10^{-12}$ M

6.02×10^{23} molecules is Avagadros number (number of molecules in a mole), the factor of 10^3 is to convert 1 ml to 1 litre (volume unit used for expressing molarity).

1×10^{-12} M (number of molecules) $\div$ 1×10^{-11} M (reciprocal of affinity constant) = 0.1K

Similarly, if the area is 10^4 μm^2 (0.01mm^2), and the other parameters remain unchanged then the antibody concentration is calculated as 0.01K

The solution for F in equation 4.2 is given by;

$$F = \frac{1+\frac{1}{[Ab]}+\frac{[Ag]}{[Ab]}-\sqrt{(1+\frac{1}{[Ab]}+\frac{[Ag]}{[Ab]})^2-4\times\frac{[Ag]}{[Ab]}}}{2} \quad (4.4)$$

Plots showing values of F at antibody concentrations between 0.0001K and 10K for several different antigen concentrations are shown in Figure 4.1. From these plots it can be seen that at antibody concentrations below 0.01K that for all practical purposes, the fractional occupancy F is dependent solely upon the antigen concentration.

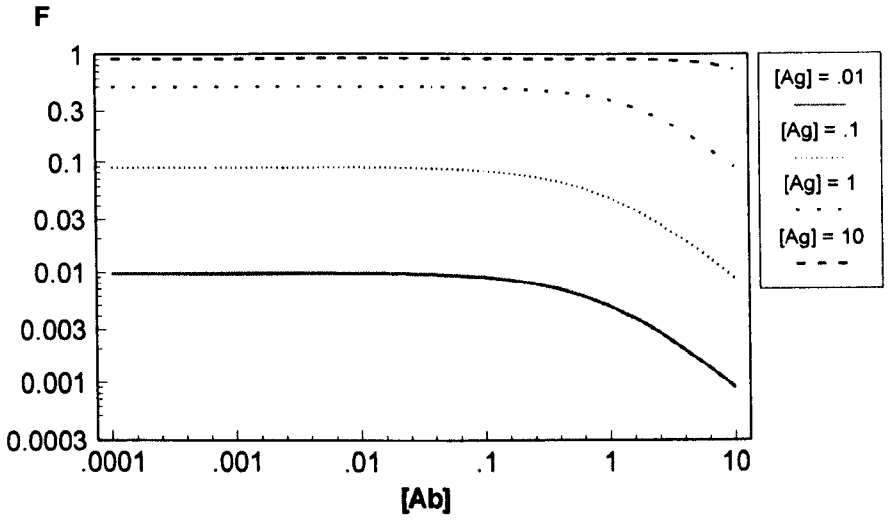


Figure 4.1 Fractional antibody binding-site occupancy (F) related to antibody concentration for several different antigen concentrations. All concentrations are in unit of 1/K. For antibody concentrations [Ab] less than approximately 0.01/K, F is governed solely by [Ag] and is essentially unaffected by variations in [Ab].

The percentage of antigen bound by the antibody (i.e.depletion of antigen from solution) is given by Equation 4.5 (from rearrangement of Equation 4.4) and plots showing the percentage of antigen bound at antibody concentrations between 0.0001K and 10K for several different antigen concentrations are shown in Figure 4.2. Note that for antibody concentrations of less than approximately 0.01/K, the percentage of antigen bound is less than 1%.

$$\%\ Antigen\ bound =$$

$$(((1+\frac{1}{[Ab]}+\frac{[Ag]}{[Ab]}-\sqrt{(1+\frac{1}{[Ab]}+\frac{[Ag]}{[Ab]})^2-4\times\frac{[Ag]}{[Ab]}}\,)\times[Ab])/2/[Ag])\times 100 \qquad (4.5)$$

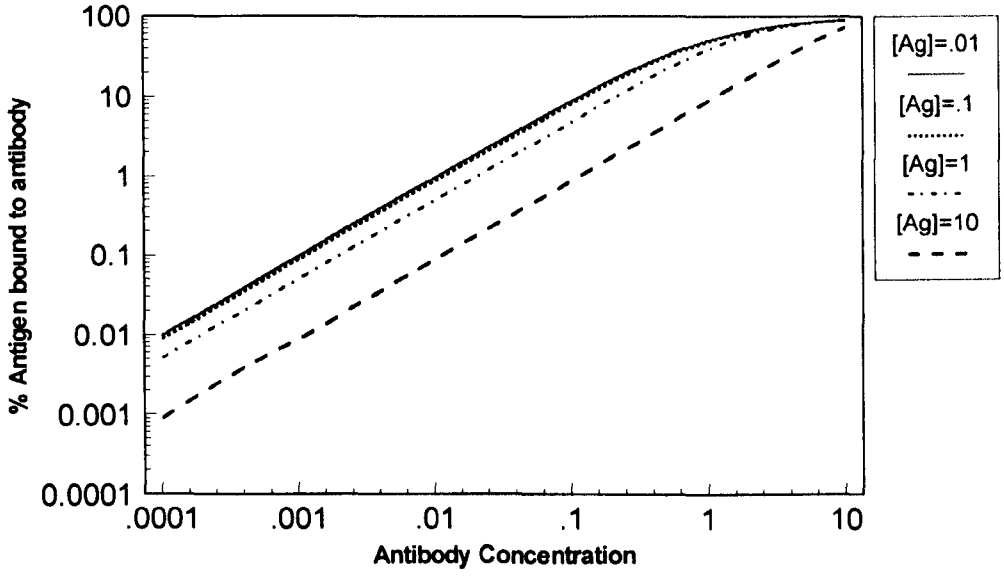


Figure 4.2 Percentage of antigen bound to antibody related to antibody concentration for several different antigen concentrations. All concentrations are in unit of 1/K. For antibody concentrations [Ab] less than approximately 0.01/K the percentage binding of the analyte is less than 1%.

It has previously been shown that a decrease in the sensor area and therefore antibody concentration (Figure 4.3a), can result in an increase in the specific/non-specific signal ratio, assuming that the "field of view" of the detecting instrument is restricted to the area on which the sensor antibody is deposited.[294] However, if the amount of capture antibody is reduced either by decreasing the antibody coated area (Figure 4.3b), or the density of antibody coating (Figure 4.3c) whilst the field of view remains constant then the signal to noise ratio (S_{sb} / S_{nsb}) ratio falls.

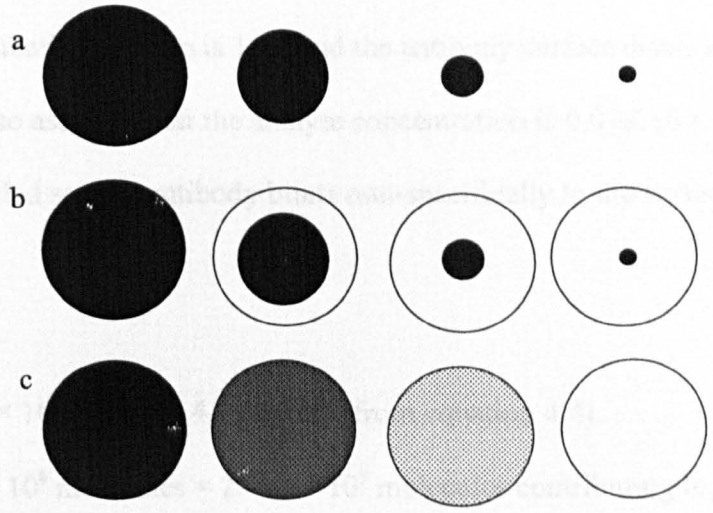


Figure 4.3 Diagram demonstrating changes in field of view, antibody-coated area and coated antibody density. Shading indicates antibody-coated area (intensity of shading represents density of antibody coated onto surface). In (a) and (c) the antibody-coated area is equivalent to the field of view, in (b) the unshaded outer circle represents the field of view. In (a), both the antibody-coated area and the field of view are reduced which leads to increased signal / noise ratio. In (b) the antibody coated area is reduced whilst the field of view remains constant, in this case the signal / noise ratio is decreased. In (c) the antibody coated area and the field of view remain constant, but the coated antibody density decreases, again this results in a decreased signal / noise ratio.

The following calculations and tables demonstrate the effect of the changes in antibody coated area, antibody density and field of view upon the specific/ non-specific signal ratio.

The number of molecules specifically bound is given by;

$F \times$ number of antibody molecules

The specific/non-specific signal ratio is given by

$$\frac{\text{Fractional occupancy (F)} \times \text{coated antibody density (molecules}/\mu m^2) \times \text{area } (\mu m^2)}{\text{non-specifically bound labelled antibody density (molecules}/\mu m^2) \times \text{area } (\mu m^2)} \qquad (4.6)$$

The fractional occupancy (F) is obtained from equation 4.4.

For example if one assumes that as before, an antibody has an affinity constant (K) of 10^{11} M^{-1}, the total incubation volume is 1 ml and the antibody surface density is 6000 molecules / μm^2, but one also assumes that the analyte concentration is 0.01K (6×10^7 molecules / ml) and that the labelled second antibody binds non-specifically to the surface at 1 molecule / μm^2.

For an area of 1×10^6 μm^2, F = 4.99×10^{-3} (from equation 4.4).

$4.99 \times 10^{-3} \times 6 \times 10^9$ molecules = 2.994×10^7 molecules contributing to the specific signal.

Non-specific binding of the labelled second antibody is 1 molecule / μm^2, therefore there are 1×10^6 molecules contributing to the non-specific signal.

Therefore the specific / non-specific signal ratio = $\frac{2.994 \times 10^7}{1 \times 10^6} = 29.94$

Similarly for an area of 1×10^5 μm^2, F =9.02×10^{-3}, resulting in a specific/non-specific signal ratio of 54.1, and for an area of 1×10^5 μm^2, F =9.80×10^{-3}, resulting in a specific/non-specific signal ratio of 58.8

If the reduction in the antibody-coated area were not accompanied by a corresponding reduction in the detecting instrument's field of view, there would not be a decrease in the background produced by non-specifically bound labelled antibody, and the specific / non-specific signal ratio would be adversely affected. This is demonstrated by calculated examples shown in Table 4.1. In this table it can be seen that if the field of view is equivalent to the area of antibody deposition, that as the size of this area decreases, the specific / non-specific signal ratio increases. If the area of antibody deposition is increased so that it is larger than the field of view, the specific / non-specific signal ratio remains unchanged, but if the area of antibody deposistion is decreased so that it is smaller than the

field of view, the specific / non-specific signal ratio decreases. The implication of this is that if one is working with instrumentation whose field of view can not be decreased below a certain area, it may be beneficial to ensure that the antibody-coated surface covers this entire area, the larger area's advantage of a proportionally smaller signal contribution by non-specifcally bound molecules outweighing the improved fractional occupancy of smaller areas.

	Specific/Non-specific signal ratio		
Area of antibody deposition μm^2	1×10^6	1×10^5	1×10^4
Field of view Area μm^2			
1×10^6	29.94	5.41	0.59
1×10^5	54.1	54.1	5.88
1×10^4	58.8	58.8	58.8

Table 4.1 Examples of specific / non-specific signal ratios for differently antibody deposition and field of view areas.

In table 4.2 it can be seen that for a given field of view (assumed equivalent to the area of antibody deposition), that as the antibody surface density decreases, so does the specific / non-specific signal ratio. It should also be noted that as the antibody surface density decreases the advantages of using a smaller field of view are greatly diminished.

	Specific/Non-specific signal ratio		
Field of View μm^2(equivalent to area of antibody deposition)	1×10^6	1×10^5	1×10^4
Antibody surface density molecules/μm^2			
6,000	29.94	54.1	58.8
600	5.41	5.88	5.93
60	0.59	0.59	0.59

Table 4.2 Examples of specific / non-specific signal ratios for different antibody deposition and antibody surface densities.

4.2.1. Influence of working electrode area as proportion of total area on the specific /non-specific signal ratio.

When measuring analytes in solution amperometrically, the sensor's "field of view" is not restricted to the area on which the sensor antibody is immobilised. The non-specific signal can have a number of constituents in addition to the non-specific binding of the second antibody-enzyme conjugate to the sensing area itself. Signal generating molecules that are bound to the surrounding area also need to be considered, and mass transport of electroactive species between electrodes can occur.

If one was to consider a case where the field of view encompasses the total surface and where diffusion was instantaneous, then the effect (for a 2 electrode system) of an electrode's size (expressed as a fraction of the entire surface) upon the specific signal (S_{sb})/non-specific signal (S_{nsb}) ratio is given by equation 4.7. The specific/non signal ratio is dependent upon the electrode area (A_e), the area of other electrodes within the system (A_o), the total area (A_t), the density of signal producing molecules specifically bound to electrode (D_{es}) and the density of signal producing molecules non-specifically bound to the electrode (D_{en}), other electrodes in the system (D_{on}) and to areas that are not electrodes (D_{nn}).

$$\frac{S_{sb}}{S_{nsb}} = \frac{(Ae.Des)}{(Ae.Den+(Ao.Don)+(A_t-Ae-Ao).Dnn)} \quad (4.7)$$

For equation 4.7 to apply for a system for any number (n) of electrodes the term $A_o.D_{on}$ is replaced with the term $\Sigma(A_o.D_{on})_{1..n-1}$. Similarly the term $(A_t-A_e-A_o)$ is replaced by $(A_t-A_e-\Sigma(A_o)_{1..n-1})$, resulting in equation 4.8.

$$\frac{S_{sb}}{S_{nsb}} = \frac{(Ae.Des)}{(Ae.Den+\Sigma(Ao.Don)_{1..n-1}+(A_t-Ae-\Sigma(Ao)_{1..n-1}).Dnn)} \quad (4.8)$$

The effect of Equation 4.8 is that as the electrode size (expressed as a fraction of total area) decreases there is a corresponding reduction in the specific/non-specific signal ratio. This is shown graphically in Figure 4.4, for a number of simulated examples.

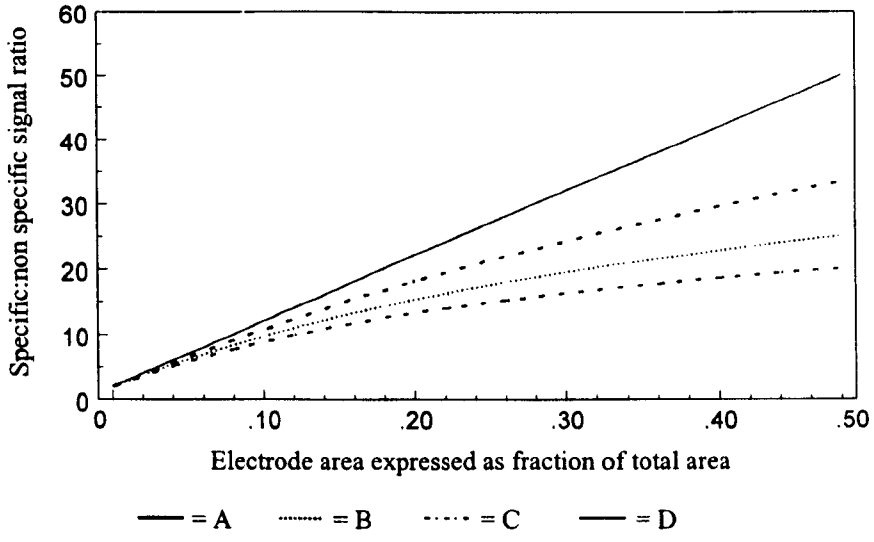


Figure 4.4 Theoretical plot of specific/non-specific signal ratio versus electrode size for several systems with differing degrees of non-specific binding in a 2 electrode system. For curve 'A' non-specific binding to both electrodes and non electrode area is 1% of specific binding to electrode, for curve 'B' non-specific binding to both electrodes and non electrode area is 2% and 1% respectively, for curve 'C' binding to electrode 1, electrode 2 and non electrode area is 2%, 1% and 1% respectively and for curve 'D' binding to electrode 1, electrode 2 and non electrode area is 2%, 3% and 1% respectively

If a stationary electrode is immersed in an unstirred electrolytic solution and a potential is applied, provided that electroactive substances that can undergo oxidation or reduction are present in the electrolytic solution, charge transfer will occur at the electrode/liquid interface. Reduction involves electron transfer from the electrode to the electroactive substance, whilst in oxidation electrons are transferred from the electroactive substance to the electrode. As this occurs, concentrations of substances at the electrode surface start to change, and diffusive mass transport commences.

Dependent upon the size of the electrode and the volume of the solution three limiting cases of diffusion can be described.[298] The first case is an electrode in a thin-layer cell where the cell volume/ electrode surface area ratio is very low. In this case mass transport within the cell is negligible, and there is no diffusion gradient. In the second case, that of a macroelectrode (not in a thin layer cell), the cell volume/electrode surface area ratio is much larger and one observes planar diffusion. In the final case when one reduces the size of the electrode so that it has an extremely small surface area (diameter < 20 μm), the conditions change and the diffusion process becomes dependent upon the size and geometry of the electrode. For disk ultramicroelectrodes the spatial diffusion field that develops is hemispherical. These different types of diffusion are illustrated in Figure 4.5.

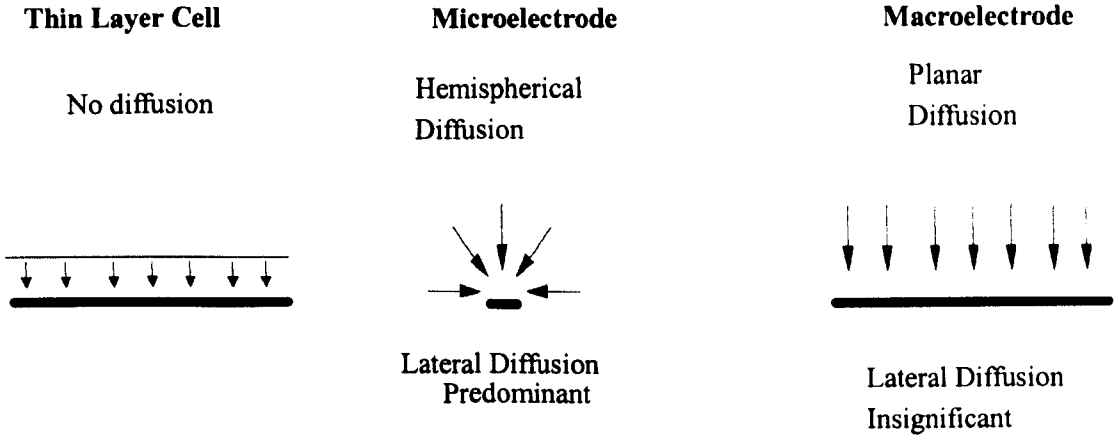


Figure 4.5 Transverse sections of a thin layer cell, microelectrode and a macroelectrode demonstrating differences in diffusion. In the thin layer cell the available transport paths are short and no diffusion gradient develops. In the microelectrode a spatial diffusion field develops (in the case of a disk electrode a hemispherical field). For the macroelectrode, planar diffusion occurs.[298]

As mentioned in Chapter 3, mass transport of electroactive species consists of three components, diffusion, migration and convection. Mass transport can be limited to just the diffusion term if the solution is quiescent (eliminating convection) and has a relatively high concentration of supporting electrolyte (eliminating migration).

As one decreases the size of an electrode, to the point of it becoming an ultramicroelectrode, then time-dependent changes in mass transport become evident. These changes involve the transformation of one-dimensional planar diffusion fields into spatial fields, with a radial diffusive component (parallel to the electrode surface) being added to the usual axial diffusion. In mathematical terms diffusion is described by Fick's Laws, which vary according to electrode geometry.[298] The equation for planar diffusion at a macroelectrode is shown in Equation 4.9, whilst that for hemispherical diffusion at a disk electrode is shown in Equation 4.10.

$$\frac{\delta c}{\delta t} = D\frac{\delta^2 c}{\delta x^2} \quad (4.9)$$

$$\frac{\delta c}{\delta t} = D\left(\frac{\delta^2 c}{\delta r^2} + \frac{1}{r}\frac{\delta c}{\delta r} + \frac{\delta^2 c}{\delta z^2}\right) \quad (4.10)$$

The increased diffusion rate of species to ultramicroelectrodes leads to a number of analytical advantages such as very high current densities (in spite of small currents), rapidly achieving a time independent stationary state, and a large drop in ohmic resistance due to the resistance of the solution.[298-300] However, in constructing arrays of ultramicroelectrodes for multianalyte analysis, the hemispherical diffusion field can be problematical. The contribution to the observed current due to signal producing molecules that are non-specifically bound to areas other than the electrode (terms D_{on} and D_{nn} in equations 4.7 and 4.8) are greatly increased, resulting in a poorer specific/non-specific signal ratio. This in turn will lead to poorer sensitivity and reduced discrimination.

The formation of the hemispherical diffusion field effectively means that the field of view of the detecting instrument is not limited to the area on which the sensor antibody is deposited and the specific / non-specific signal ratio (from equation 4.6) would be adversely affected, as shown in Table 4.1.

In Table 4.1 it can be seen that if the field of view is equivalent to the area of antibody deposition, that as the size of this area decreases, the specific / non-specific signal ratio increases. If the area of antibody deposition is increased so that it is larger than the field of view, the specific / non-specific signal ratio remains unchanged, but if the area of antibody deposition is decreased so that it is smaller than the field of view, the specific / non-specific signal ratio decreases. As stated earlier, the implication of this is that if one is working with instrumentation whose field of view can not be decreased below a certain area (such as hemispherical diffusion at ultramicroelectrodes), it may be beneficial to ensure that the antibody-coated surface covers this entire area, the larger area's advantage of a proportionally smaller signal contribution by non-specifically bound molecules outweighing the improved fractional occupancy of smaller areas.

4.3 Materials

Positive S1400-31 photoresist and Microposit developer were obtained from Shipley Europe, Coventry, England. Glucose oxidase (EC 1.1.3.4, 20 U mg^{-1}), sodium glutamate, ferrocenemonocarboxylic acid, N-acetyl-l-cysteine, and1-ethyl-3-(3-dimethylaminopropyl) - carbodiimide were obtained from Sigma, Poole, England.

Glutamate oxidase (EC 1.4.3.11.5, 16 U mg^{-1}) was kindly provided by Yamasa Shoyu, Chiba, Japan.

Silicon wafers were obtained from Shin Etsu, Livingston, Scotland. NiChrome (80:20 Ni:Cr) and gold were obtained from Goodfellow Cambridge Ltd, Cambridge, England. Neutravidin™ and casein were obtained from Pierce Warriner, Chester, England. Photobiotin was obtained from Vector Laboratories, Peterborough, England.

A Bioanalytical Systems (BAS) RE4 Ag/AgCl reference electrode was obtained from Biotech Instruments Ltd, Luton, England.

Instrumentation used comprised a EG&G 273A potentiostat (EG & G, Sunninghill, England), Bioanalytical System CV-37 potentiostats (Biotech Instruments Ltd, Luton, England), a Goerz SE120 dual channel chart recorder (Belmont Instruments, Glasgow, UK), a 100W HB-10101AF super high pressure mercury vapour lamp (Nikon, Tokyo, Japan) and a photolithographic mask aligner (HTG San Jose, California, USA).

4.4. Methods

4.4.1 Method for fabrication of electrodes and electrode arrays using photolithographic lift-off techniques.

Positive S1400-31 photoresist was spun onto 10 cm diameter silicon wafers at 4000 rpm for 30 s and then baked at 90°C for 30 minutes. The resist was then illuminated by light for 12 s from a mask aligner through a photolithographic mask containing relief images of the electrode arrays (Figures 4.6 to 4.8). Exposed resist was removed by immersion in Microposit developer for 75 s followed by a rinse in reverse osmosis (RO) water. NiChrome and gold were deposited on the surface to a thickness of 10nm and 100nm respectively, using a modified Edwards 12E High Vacuum evaporation unit. The remaining photoresist was removed by immersion in acetone for 20 minutes, lifting off the metal deposited on it, leaving the silicon wafer with the metallic electrode arrays on its surface. S1400-31 photoresist was spun onto the surface again, and baked at 90°C as detailed above. For the electrodes shown in Figures 4.6 and 4.7, all areas of the surface apart from the connecting tracks were exposed to light through a second mask (this was so that the connecting track did not form part of the electrode, and to allow investigations into the effect of specifically and non-specifically bound enzymes to the area surrounding the electrode). For the electrodes shown in Figures 4.8, electrodes and bonding pads (but not connecting tracks) were exposed to light through a second mask. Exposed resist was removed as detailed above, and the wafer was then baked at 120°C for 120 minutes, to harden the remaining photoresist that acted as an electrical insulator.

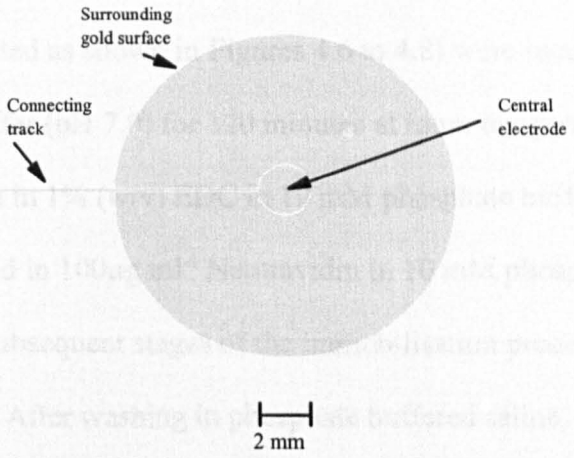


Figure 4.6 Diagrammatic representation of a photolithographically fabricated gold electrode with an electrode 2 mm in diameter and surrounded by a gold non-electrode surface. Details of the fabrication procedure are provided in the text (Section 4.4.1)

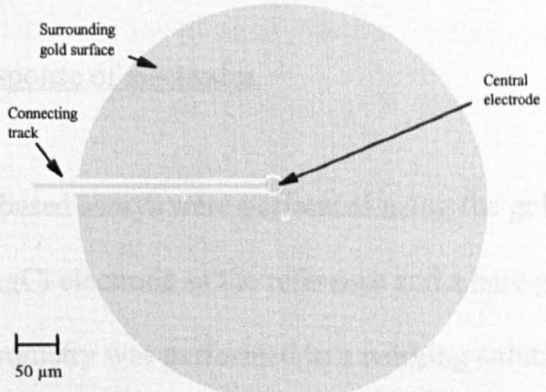


Figure 4.7 Diagrammatic representation of an photolithographically fabricated gold electrode with an electrode 10 µm in diameter and surrounded by a gold non-electrode surface. Details of the fabrication procedure are provided in the text (Section 4.4.1)

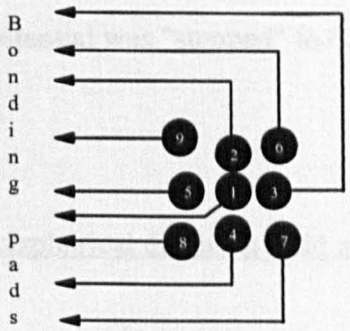


Figure 4.8 Diagrammatic representation of a photolithographically fabricated electrode array with electrodes either 1 mm or 10 µm in diameter. Details of the fabrication procedure are provided in the text (Section 4.4.1)

4.4.2. Avidin-photobiotin immobilisation procedure.

Electrodes (fabricated as shown in Figures 4.6 to 4.8) were incubated in 2 mM NAC in 10 mM phosphate buffer (pH 7.0) for 120 minutes at room temperature, followed by 120 minutes incubation in 1% (w/v) EDC in 10 mM phosphate buffer (pH 7.0). The electrodes were then incubated in 100μg ml^{-1} Neutravidin in 10 mM phosphate buffer (pH 7.0) for 16 hours at 4°C. All subsequent stages of the immobilisation procedure were performed at room temperature. After washing in phosphate buffered saline, pH 7.4 (PBS), the electrodes were first incubated in 10 mg ml^{-1} casein in PBS for 60 minutes and then in 10 μg ml^{-1} long arm photobiotin in PBS, for 20 minutes in the dark.

4.4.3. Amperometric response of electrodes.

Amperometric enzyme based assays were performed using the gold electrode as a working electrode, with an Ag/AgCl electrode as the reference and a bare platinum flag as a counter electrode. Chronoamperometry was performed in a working solution containing 10 mM sodium phosphate, 140 mM NaCl, 25 mM KCl, pH 7.0. Dependent upon the experiment, glucose and / or glutamate could be added to this working solution to provide predetermined concentrations. Initially, the working electrode was poised at a potential of 0V for 10 seconds, after which the potential was "stepped" to 650mV for 120 seconds at which time the current was recorded.

4.4.4 Investigation into effect of hemispherical diffusion field upon response of ultramicroelectrodes.

4.4.4.1 Enzyme immobilisation procedure

Avidin and photobiotin were immobilised on electrodes (as shown in Figures 4.6 and 4.7) as described in Section 4.4.2. After washing in PBS the wafer was covered with 10 μg ml^{-1}

glucose oxidase (GOD) and the desired areas (as shown in Table 4.3) were exposed to light as described in Section 2.3.1. After washing in PBS, the entire wafer was exposed to light for 15 minutes in the presence of 10 mg ml^{-1} casein in PBS, and again washed in PBS. Areas of electrodes (as shown in Table 4.3) were then exposed to deep UV radiation ($\lambda <$ 200 nm, irradiance = 200 mW cm^{-2}) in order to inactivate any GOD that had been non-specifically bound. The areas containing specifically and non-specifically bound GOD are shown in Table 4.4. After the enzyme immobilisation procedure was complete a plastic ring was attached to the substrate to allow solutions to be placed in the cell. The prepared electrodes were stored in PBS until use.

Electrode	Area exposed to light in presence of GOD during patterning process	Area exposed to DUV following patterning process.
A	None	Entire surface
B	None	Entire surface apart from electrode
C	None	Electrode only
D	None	None
E	Electrode only	Entire surface apart from electrode
F	Electrode only	None
G	Entire surface apart from electrode	Electrode only
H	Entire surface apart from electrode	None
I	Entire surface	None

Table 4.3 Areas of sensor surfaces exposed to light in presence of GOD during patterning process and those areas exposed to DUV radiation following the patterning process. Exposure to light during the patterning process resulted in specific immobilisation of GOD, whilst in areas irradiated with DUV light the GOD was rendered non-functional.

		Area with non-specifically bound GOD			
		None	Electrode	Other than electrode	All
Area with specifically bound GOD	None	A	B	C	D
	Electrode	-	E	-	F
	Other than electrode	-	-	G	H
	All	-	-	-	I

Table 4.4 Areas of sensor surfaces containing specifically and non-specifically bound GOD. Note that it was not possible to have an area with specifically bound GOD in the absence of non-specifically bound GOD.

4.4.4.2. Amperometric Analysis

Chronoamperometry was performed as described in Section 4.4.3 using solutions containing 100 mM glucose. Amperometric analysis was performed using 2mm and 10 μm diameter electrodes (Figures 4.6 & 4.7) with a +650 mV potential (versus Ag/AgCl) applied to the electrode alone, and with a potential of +650 mV also applied to the surrounding gold area from a second independent potentiostat. Results are shown in Section 4.5.1.

4.4.5 Investigation into analytical "cross-talk" caused by mass transport of electrochemical species generated at one electrode to other electrodes in an array.

4.4.5.1 Enzyme immobilisation procedure

Avidin and photobiotin were immobilised on electrodes (as shown in Figure 4.8) as described in Section 4.4.2. After washing in PBS the wafer was covered with 10 μg ml^{-1} GOD and the generator electrode (electrode 1 in Figure 4.8) was exposed to light and then blocked with casein as described in Section 4.4.4.1. Areas other than the generator electrode were exposed to DUV radiation, fitted with a plastic ring and stored as described in Section 4.4.4.1.

4.4.5.2 Amperometric analysis

Chronoamperometry was performed as described in Section 4.4.3. A series of experiments were performed, applying a potential of +650 mV versus the Ag/AgCl reference electrode to each of the electrodes in turn. Upon obtaining a stable baseline reading, glucose was added to give a final concentration of 100 mM. The current was recorded 120 seconds after the addition of the glucose. This series of experiments were then repeated, applying a potential of 650mV to the generator electrode as well as the monitoring electrode. Plots of current versus distance of measuring electrode from generator electrode are shown in Section 4.5.2.

4.4.6 Construction of multianalyte enzymic biosensor using ultramicroelectrodes.

The analytes were chosen were glucose and glutamate as oxidase enzymes (whose action resulted in the formation of H_2O_2) were available for these 2 substances.

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\textit{glucose oxidase}} \text{Gluconolactone} + H_2O_2$$

$$\text{Glutamate} + O_2 \xrightarrow{\textit{glutamate oxidase}} \text{2-Oxoglutarate} + NH_3 + H_2O_2$$

4.4.6.1 Electrode fabrication.

Electrodes were photolithographically fabricated as described in Section 4.4.1. The fabricated electrodes were 10 μm in diameter and located 150 μm apart. Tracks 3 μm wide (which were electrically insulated by the use of photoresist) connected the electrodes to bonding pads, where connections were made to the potentiostats.

4.4.6.2 Enzyme immobilisation procedure.

Avidin and photobiotin were immobilised on electrodes as described in Section 4.4.2. After washing in PBS the wafer was covered with 10 μg ml^{-1} glutamate oxidase (GLOD) for 15 minutes and one of the electrodes was exposed to light as described in Section 4.4.4.1. After washing in PBS the wafer was covered with 10 μg ml^{-1} GOD and the other electrode was exposed to light 15 minutes The surface was then blocked with casein as described in Section 4.4.4.1. Areas of the surface other than the electrodes were exposed to DUV radiation, fitted with a plastic ring and stored as described in Section 4.4.4.1.

4.4.6.3 Amperometric analysis.

Chronoamperometry was performed as described in Section 4.4.3. with solutions containing varying concentrations of glucose or glutamate. Results are shown in Section 4.5.3.

4.4.7 Construction of multianalyte immunosensor using ultramicroelectrodes.

4.4.7.1 Electrode Fabrication

Electrodes were photolithographically fabricated as described in Section 4.4.1. The fabricated electrodes were 10 μm in diameter and were fabricated in pairs situated 1mm apart (centre to centre), individual electrodes were as shown in Figure 4.7. Tracks 3 μm wide(which were electrically insulated by the use of photoresist) connected the electrodes to bonding pads where attachments were made to the potentiostats.

4.4.7.2 Antibody immobilisation

Avidin and photobiotin were immobilised on electrodes as described in Section 4.4.2. After washing in PBS the wafer was covered with 10 μg ml^{-1} goat anti-rabbit IgG for 15 minutes and one of the electrodes was exposed to light as described in Section 4.4.4.1. After

washing in PBS the wafer was covered with 10 μg ml^{-1} goat anti-rat IgG and the other electrode was exposed to light 15 minutes The surface was then blocked with casein as described in Section 4.4.4.1. Areas of the surface other than the electrodes were exposed to DUV radiation, fitted with a plastic ring and stored as described in Section 4.4.4.1.

Antibodies were also immobilised to electrodes in the way described above, but instead of just the electrodes being exposed to light during the patterning process, the area of patterned gold surrounding the electrodes (Figure 4.7) was also exposed, thus increasing the area of specifically bound antibody.

4.4.7.3 Immunoassay procedure.

Electrodes were incubated in solutions of PBS or PBS containing 10 μg ml^{-1} rat IgG and / or 10 μg ml^{-1} rabbit IgG for 60 minutes at room temperature. Following washing with PBS, electrodes were incubated in 10 μg ml^{-1} goat anti-rat IgG-HRP conjugate and 10 μg ml^{-1} goat anti-rabbit IgG-HRP conjugate for 60 minutes at room temperature

Simultaneous assessment of HRP activity was performed chronoamperometrically using two Bioanalytical System CV-37 potentiostats and a Goerz SE120 dual channel chart recorder. Activity was determined by measuring the current produced at 120 s in the presence of 10 mM hydrogen peroxide, and 0.2 mM ferrocenemonocarboxylic acid in 50 mM phosphate buffer containing 50 mM KCl, pH 7.4, with a potential of +150 mV applied to the working electrode versus the Ag/AgCl reference electrode.[290]

4.5 Results and Discussion

4.5.1 Investigation into effect of hemispherical diffusion field upon response of ultramicroelectrodes.

The results shown in Table 4.5 demonstrate that the observed current for 2 mm diameter electrodes was predominantly a result of electroactive species being generated at the electrode surface. The currents observed when GOD has been specifically immobilised on the electrode were significantly higher than those where GOD had been specifically immobilised on the surrounding gold but not on the electrode itself, and for those where only non-specific binding was present. The currents observed where GOD had been specifically immobilised on the surrounding gold but not on the electrode itself, were slightly higher than those where only non-specific binding was present, indicating that some diffusion to the electrode of H_2O_2 generated in the surrounding area was occurring. These results also indicate that the effect of non-specifically bound GOD on observed currents is small.

The results shown in Table 4.6 demonstrate that for 10 μm diameter electrodes non-specific binding of GOD to the gold surface surrounding the electrode can produce a very significant current at the working electrode. This is as the H_2O_2 produced in this surrounding area diffuses to the working electrode, and this non-specific signal is similar in magnitude to that obtained when GOD is specifically immobilised at the working electrode. These results indicate that when using ultramicroelectrode arrays for multianalyte assays the effect of non-specific binding (in particular to the surrounding surface) can be critical.

The results in Tables 4.5 and 4.7 indicate that if in the presence of glucose, a potential of +650 mV vs. Ag/AgCl was applied to the area of gold surrounding the 2 mm diameter

electrode as well as to the electrode itself, only a small decrease in current from the working electrode was observed compared with when the potential was applied to the electrode alone. This was as the diffusion at the 2 mm diameter electrode is predominantly planar with only a relatively small lateral component, which is further demonstrated by the relatively small current observed when there is no functional GOD deposited on the electrode itself, but functional GOD is present on the surrounding gold surface.

The results in Tables 4.6 and 4.8 indicate that if in the presence of glucose, a potential of +650 mV vs. Ag/AgCl was applied to the area of gold surrounding the 10 μm diameter electrode as well as to the electrode itself, the resultant current from the working electrode was, in most cases, indistinguishable from the signal when no glucose was present. This was because the surrounding gold was acting as a macroelectrode and eliminated the hemispherical diffusion field surrounding the ultramicroelectrode. The only exception was that where GOD was specifically immobilised on the electrode, and any non-specifically bound GOD in the surrounding area was rendered non-functional by DUV irradiation. In this case a hemispherical diffusion of glucose to the electrode can occur.

These results suggest that for multianalyte sensors employing ultramicroelectrodes one should specifically immobilise the biological element over the surface surrounding the electrode as well as on the electrode itself. This is so that the specific signal is being generated over the entire "field of view" (i.e. the hemispherical diffusion field), thus improving the specific/nonspecific signal ratio as discussed earlier in this chapter by increasing S_{sb} in Equations 4.7 and 4.8. An alternative is to render any molecules that are non-specifically bound to the area surrounding the electrode non-functional by techniques such as DUV irradiation, and thus decreasing S_{nsb} in Equations 4.7 and 4.8.

		Current nA			
		Area with non-specifically bound GOD			
		None	Electrode	Other than electrode	All
Area with specifically bound GOD	None	63	76	78	84
	Electrode	-	358	-	346
	Other than electrode	-	-	118	122
	All	-	-	-	347

Table 4.5 Currents obtained from 2 mm diameter electrodes in the presence of glucose when no potential was applied to the surrounding gold surface. Chronoamperometry was performed as described in Section 4.4.4.2.

		Current pA			
		Area with non-specifically bound GOD			
		None	Electrode	Other than electrode	All
Area with specifically bound GOD	None	23	47	102	99
	Electrode	-	243	-	265
	Other than electrode	-	-	233	238
	All	-	-	-	258

Table 4.6 Currents obtained from 10 μm diameter electrodes in the presence of glucose when no potential was applied to the surrounding gold surface. Chronoamperometry was performed as described in Section 4.4.4.2.

		Current nA			
		Area with non-specifically bound GOD			
		None	Electrode	Other than electrode	All
Area with specifically bound GOD	None	54	69	57	67
	Electrode	-	338	-	341
	Other than electrode	-	-	71	77
	All	-	-	-	329

Table 4.7 Currents obtained from the 2 mm diameter electrodes in the presence of glucose when a potential of +650 mV was applied to the surrounding gold surface as well as to the working electrode.

Chronoamperometry was performed as described in Section 4.4.4.2.

		Current pA			
		Area with non-specifically bound GOD			
		None	Electrode	Other than electrode	All
Area with specifically bound GOD	None	19	23	18	22
	Electrode	-	198	-	23
	Other than electrode	-	-	30	22
	All	-	-	-	31

Table 4.8 Currents obtained from the 10 μm diameter electrodes in the presence of glucose when a potential of +650 mV was applied to the surrounding gold surface as well as to the working electrode.

Chronoamperometry was performed as described in Section 4.4.4.2.

4.5.2 Investigation into analytical "cross-talk" caused by mass transport of electrochemical species generated at one electrode to other electrodes in an array.

The data presented in Figures 4.9 and 4.10 indicates that unless electrodes are in very close proximity the contribution of electroactive species generated at one electrode to the observed response at another is minimal. It should be noted that distances in these Figures are expressed from the centre of one electrode to the centre of another. The contribution of electroactive species generated at one electrode to the observed response at another is more pronounced for the 10 μm electrodes than for the 1 mm electrodes, which reflects the increased importance of lateral diffusion for ultramicroelectrodes. That lateral diffusion is less significant for larger electrodes, is also shown by the greater proportional change in current for ultramicroelectrodes, when the generator as well as the measuring electrode is held at a potential of +650 mV vs. Ag/AgCl.

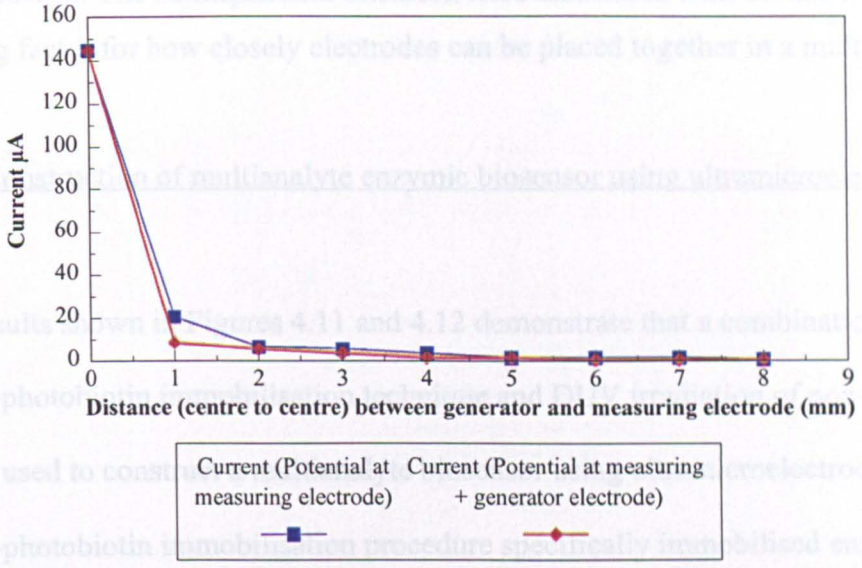


Figure 4.9 Current (background subtracted) as a function of electrode distance from the generator electrode for 1 mm diameter electrodes. Experimental details are provided in the text (Section 4.4.5)

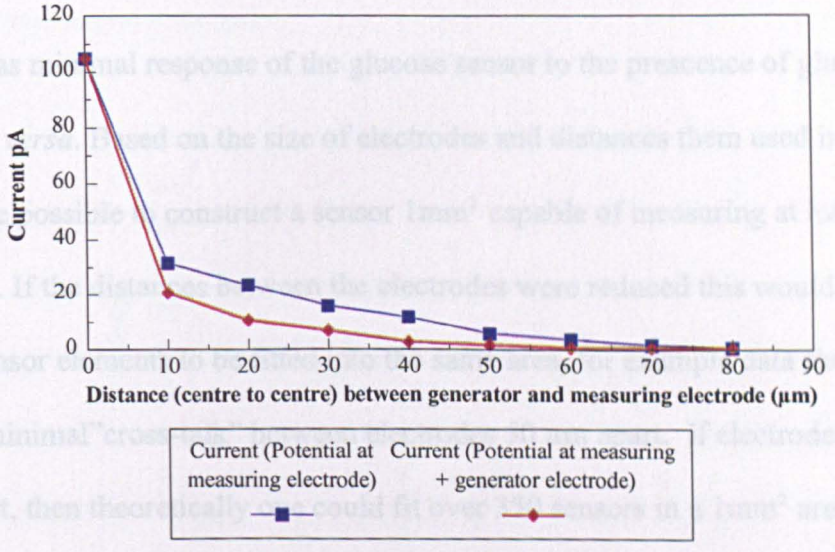


Figure 4.10 Current (background subtracted) as a function of electrode distance from the generator electrode for 10 µm diameter electrodes. Experimental details are provided in the text (Section 4.4.5)

Equations 4.7 and 4.8 show that the contribution to the non-specific signal (S_{nsb}), is dependent upon the relative area and number of other electrodes within an array. The effects are cumulative and care should be taken in the design of multianalyte electrode arrays to locate electrodes so that lateral diffusion of electrochemical species between them is negligible, particularly when using ultramicroelectrodes, where lateral diffusion

predominates. The hemispherical diffusion field associated with ultramicroelectrodes is a limiting factor for how closely electrodes can be placed together in a multianalyte system.

4.5.3 Construction of multianalyte enzymic biosensor using ultramicroelectrodes.

The results shown in Figures 4.11 and 4.12 demonstrate that a combination of the avidin-photobiotin immobilisation technique and DUV irradiation of non-electrode areas can be used to construct a multianalyte biosensor using ultramicroelectrodes. The avidin-photobiotin immobilisation procedure specifically immobilised enzymes on the electrodes, and any molecules that had non-specifically bound to non-electrode surfaces were inactivated by the use of DUV irradiation.

There was minimal response of the glucose sensor to the prescence of glutamate in solution and *vice versa*. Based on the size of electrodes and distances them used in this work it would be possible to construct a sensor $1mm^2$ capable of measuring at least 25 discrete analytes. If the distances between the electrodes were reduced this would allow significantly more sensor elements to be fitted into the same area, for example data shown in Figure 4.11 shows minimal"cross-talk" between electrodes 50 μm apart. If electrodes were located 50 μm apart, then theoretically one could fit over 350 sensors in a $1mm^2$ area, each sensor responding to a different analyte.

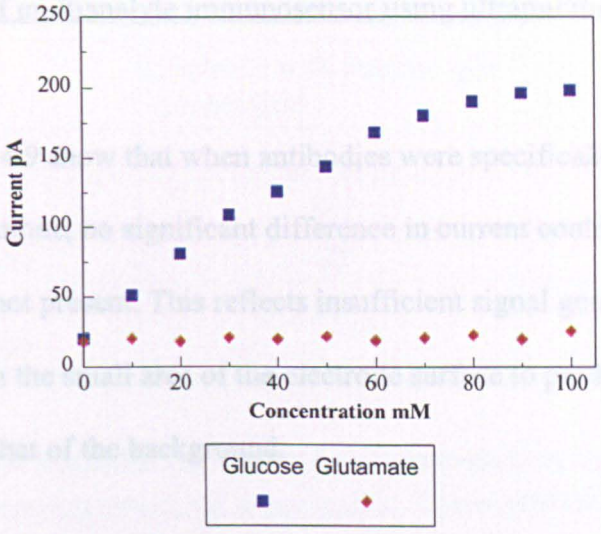


Figure 4.11 Response of the glucose oxidase electrode to solutions containing glucose and glutamate. GOD and GLOD were specifically immobilised on discrete 10 µm diameter ultramicroelectrodes using the avidin-photobiotin method. An amperometric enzyme based assay was performed using the modified gold surface as a working electrode, with an Ag/AgCl electrode as the reference. Chronoamperometry was performed as described in the text.

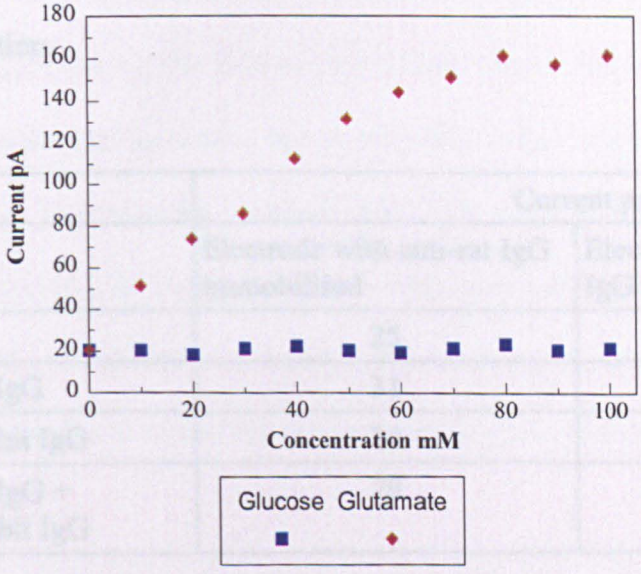


Figure 4.12 Response of the glutamate oxidase electrode to solutions containing glucose and glutamate. GOD and GLOD were specifically immobilised on discrete 10 µm diameter ultramicroelectrodes using the avidin-photobiotin method. An amperometric enzyme based assay was performed using the modified gold surface as a working electrode, with an Ag/AgCl electrode as the reference. Chronoamperometry was performed as described in the text.

4.5.4 Construction of multianalyte immunosensor using ultramicroelectrodes.

The results in Table 4.9 show that when antibodies were specifically immobilised on the ultramicroelectrode alone, no significant difference in current could be observed when antigen was or was not present. This reflects insufficient signal generating molecules (i.e. conjugate) present in the small area of the electrode surface to produce a current differentiable from that of the background.

The results in Table 4.10 show that when antibodies were specifically immobilised on the area surrounding the electrode as well as the ultramicroelectrode itself, significant differences in current could be observed when antigen was or was not present. The difference between this case and that when antibody was immobilised on the electrode alone, is due to a greatly increased amount of conjugate being bound, with a related increase in signal generation.

	Current pA	
Sample	Electrode with anti-rat IgG immobilised	Electrode with anti-rabbit IgG immobilised
PBS	25	22
10 $\mu g\ ml^{-1}$ rat IgG	31	25
10 $\mu g\ ml^{-1}$ rabbit IgG	26	28
10 $\mu g\ ml^{-1}$ rat IgG + 10 $\mu g\ ml^{-1}$ rabbit IgG	29	28

Table 4.9 Currents obtained in presence of antigens for ultramicroelectrode immunoassay when antibody was immobilised only on electrode. Experimental details are provided in the text (Section 4.4.7)

	Current pA	
Sample	Electrode with anti-rat IgG immobilised	Electrode with anti-rabbit IgG immobilised
PBS	23	24
10 μg ml^{-1} rat IgG	107	31
10 μg ml^{-1} rabbit IgG	28	94
10 μg ml^{-1} rat IgG + 10 μg ml^{-1} rabbit IgG	124	115

Table 4.10 Currents obtained in presence of antigens for ultramicroelectrode immunoassay when antibody was immobilised on surrounding area as well as on electrode. Experimental details are provided in the text (Section 4.4.7)

The qualitative immunosensor fabricated in this section of work demonstrates the feasibility of constructing multianalyte amperometric immunosensors using ultramicroelectrodes. The work presented in this chapter shows that when using ultramicroelectrodes in multianalyte assays, it is necessary take into account the field of view of the system (defined by the area of hemispherical diffusion).The field of view effects can be limited by destroying the activity of any biological molecules non-specifically bound to the surrounding area (e.g. by DUV irradiation). This provides increased sensitivity and specificity by decreasing the non-specific signal (S_{nsb} in Equations 4.7 and 4.8). Alternatively one can increase the specific signal (S_{sb} in Equations 4.7 and 4.8) by extending the area of specifically immobilised molecules to that of the area surrounding the electrode.

CHAPTER 5

And in such indexes, although small pricks

To their subsequent volumes, there is seen

The baby Figure of the giant mass

of things to come at large

William Shakespeare

Troilus and Cressida

5 Further applications of avidin-photobiotin immobilisation procedure

This chapter examines the extension of the avidin-photobiotin immobilisation technique to applications other than protein based biosensors. Initially, work was performed to demonstrate the patterning of DNA on silicon dioxide, following on from this, the avidin-photobiotin patterning procedure was used to immobilise oligonucleotides as the basis of an assay to detect a genetic polymorphism. Additionally, since the publication of the avidin-photobiotin immobilisation technique,[197] it (or variants thereof) has been employed by a number of other workers, and this work, along with some future prospects, are briefly reviewed in this chapter.

5.1 Patterning of nucleic acids

Patterning of different nucleic acids or oligonucleotides on to sensor surfaces could be useful in the construction of biosensors for the detection of a number of genetic diseases or polymorphisms. An example where this application may prove useful is in the assessment of thrombotic risk. There are several polymorphisms, associated with an increased thrombotic risk, of particular interest are those for Factor V_{Leiden}, Factor II_{Leiden} and methylene- tetrahydrofolate reductase (MTHFR).

Factor V is one of the components of the coagulation cascade (Figure 5.1) and acts as a cofactor for Factor X. Factor V is converted to its active form by the action of thrombin, and subsequently inactivated by the action of activated protein C (APC). It has been noted that in some people who are prone to thrombosis, that their Factor V is resistant to cleavage by APC.[301] It has subsequently been demonstrated that the abnormality is caused by the

replacement of arginine by glutamine at residue 506,[302] which is a major cleavage site for APC, and thus the mutation is associated with a prolonged plasma half-life of activated Factor V. The possession of this variant commonly referred to as Factor V_{Leiden}, is associated with an increased risk of thrombosis and is an important risk factor in populations of European descent, where it has an incidence of approximately 7%.[302] In heterozygotes the risk of thrombosis is increased approximately 7-fold, whereas homozygotes have about 80 times more chance of having a thrombotic episode as compared to someone with the normal Factor V gene.[302]

Similarly a mutation with the Factor II (prothrombin) gene is associated with increased risk of thrombosis.[303] Like Factor V, Factor II is a component of the coagulation cascade (Figure 5.1), and the variant protein is frequently referred to as Factor II_{Leiden} This mutation is present in about 1% of the European population and is associated with elevated prothrombin levels. It is thought that these elevated levels disturb the balance of the coagulation system and result in a tendency for inappropriate clot formation to occur.[303]

Homocysteine is a sulphur containing amino acid, that although itself not a building block for proteins, lies at an important metabolic branch point in the metabolism of methionine and cysteine as shown in Figure 5.2.[304] A mutation in MTHFR (the enzyme responsible for converting homocysteine back to methionine) is thought to be a frequent and significant cause for mild hyperhomocysteinaemia.[305] A number of large patient-control studies have shown that even mild hyperhomocysteinamia is a risk factor for thrombosis.[306,307]

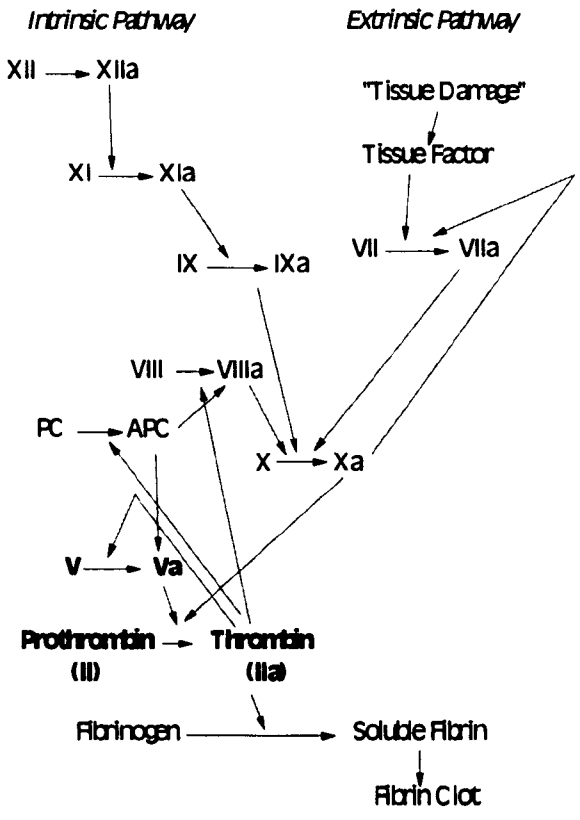


Figure 5.1 Simplified version of the coagulation cascade. Arrows in green are those catalysing conversion of clotting factors and promoting procoagulant activity. Arrows in red are inhibitory, converting an activated factor to an inactive form and thus promoting anticoagulant activity.

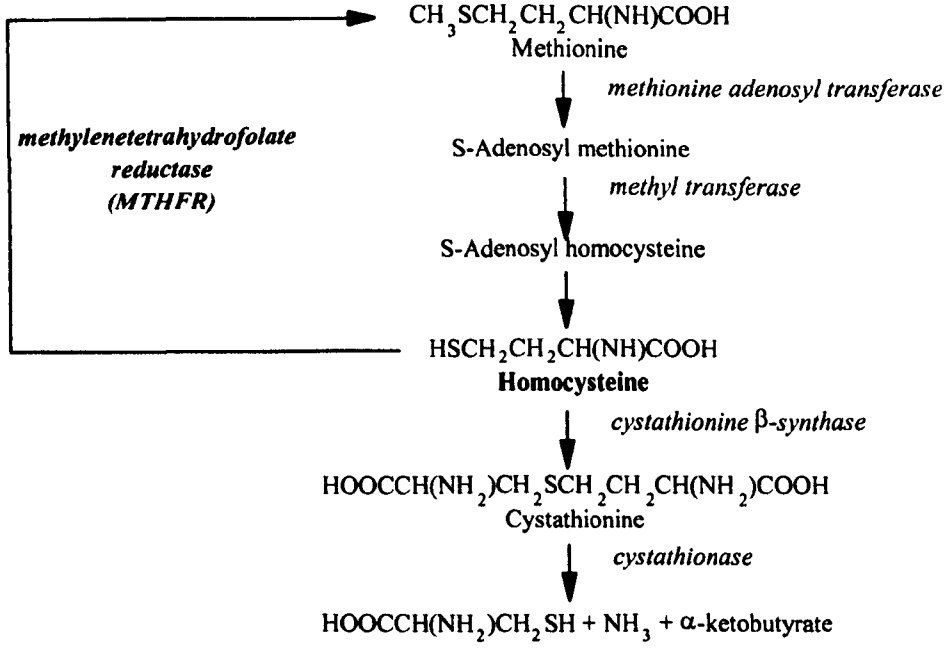


Figure 5.2 Biochemical pathways of the conversion of homocysteine to methione and cysteine.

What is particularly important about the above mentioned mutations predisposing to thrombosis, is that the risk increases cumulatively according to the number of the mutations that an individual has. For example, if an individual is heterozygotic for Factor V_{Leiden} then the risk of thrombosis is increased approximately 7-fold; if the individual is also heterozygotic for Factor II_{Leiden}, then the thrombotic risk is increased approximately 30-fold; and if the individual also has the MTHFR variant, then they are about 100 times more likely to have a thrombotic event.[308] This cumulative risk make it relevant to measure these analytes in a multianalyte system.

5.1.1 DNA amplification

In order to obtain sufficient quantities of DNA from tissue samples for genetic analysis, it is usually necessary to use a DNA amplification procedure, the most commonly used is the polymerase chain reaction (PCR). PCR relies on multiple amplifications between two oligonucleotide primers complementary to the anti-parallel DNA strands using a directionally specific DNA polymerase.[309,310] The principle of the polymerase chain reaction is shown in Figure 5.3.

PCR utilises the ability of an oligonucleotide "primer" to target a specific nucleotide sequence on denatured DNA and form an initiation site for DNA polymerase to synthesize a copy of the template. Primers are selected to be complementary to opposite strands of the DNA, and so that the sequence to be detected is present within the section between them. The action of the polymerase results in the formation of two additional DNA strands containing the primers at the 5' end. After denaturation, 4 strands of DNA are available for annealing of primers and subsequent polymerisation. The three steps of the reaction;

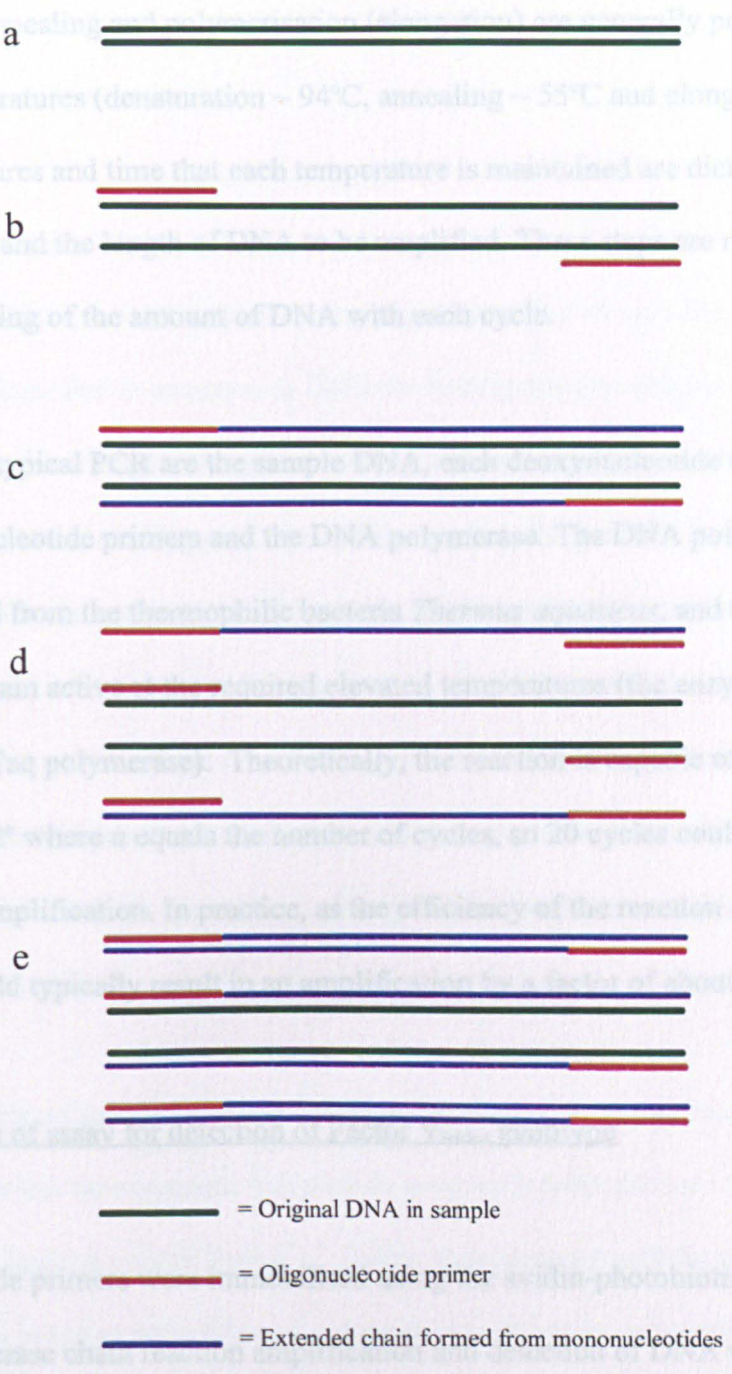


Figure 5.3 Principle of the polymerase chain reaction method of amplifying DNA. a), Sample DNA is denatured so that the complementary strands separate. b) An oligonucleotide primer anneals to the denatured DNA and c) nucleotides are incorporated into a new DNA chain by the action the action of Taq polymerase (elongation). d) A new cycle begins with denaturation of sample and newly synthesised DNA, and primers annealing to the DNA strands followed by e) elongation. The process is repeated for further cycles, with a potential doubling of DNA each time.

denaturation, annealing and polymerisation (elongation) are generally performed at three different temperatures (denaturation ~ 94°C, annealing ~ 55°C and elongation ~ 72°C). The exact temperatures and time that each temperature is maintained are dictated by factors such as sample type and the length of DNA to be amplified. These steps are repeated with a potential doubling of the amount of DNA with each cycle.

Reactants in a typical PCR are the sample DNA, each deoxynucleotide triphosphate (A, T, C, G), oligonucleotide primers and the DNA polymerase. The DNA polymerase used in PCR is derived from the thermophilic bacteria *Thermus aquaticus*, and thus allows the enzyme to remain active at the required elevated temperatures (the enzyme usually being referred to as Taq polymerase). Theoretically, the reaction is capable of amplifying DNA by a factor of 2^n where n equals the number of cycles, so 20 cycles could result in a million-fold amplification. In practice, as the efficiency of the reaction is less than 100 %, 20 cycles would typically result in an amplification by a factor of about 250,000.

5.1.2 Principle of assay for detection of Factor V_{Leiden} genotype

Oligonucleotide primers were immobilised using the avidin-photobiotin technique and in-situ polymerase chain reaction amplification and detection of DNA was undertaken.

The principle of the assay is shown in Figure 5.4. Wells of a PCR microtitre plate had one of two oligonucleotide primers attached using the avidin-photobiotin immobilisation technique. Primer 1 which was complementary to the normal Factor V genotype was immobilised in 50% of the wells whilst Primer 2 whichwa complementary to the Factor V_{Leiden} genotype was immobilised in the others. Extracted human DNA was added to the wells (each sample being added to 2 wells, one containing primer 1 and the other containing

primer 2) along with the other reactants (as described in Section 5.1), and the plate was placed in a thermal cycler.

The DNA was denatured and bound to the immobilised specific Factor V genoytype primer(s) with which it had complementary sequences. The sample DNA also bound to the biotinylated primer that is common to DNA for both genotypes (Figure 5.4a). Taq polymerase acted to elongate the primer chain by incorporation of nucleotides using the sample DNA as template (Figure 5.4b). With a new cycle, primers bound to the newly synthesised DNA chains (as well as to original DNA) and amplification continued (Figure 5.4c) until biotinylated primers were attached to all of the immobilised primers via newly synthesised DNA chains (Figure 5.4d). Streptavidin-HRP conjugate was added which attached to biotin (Figure 5.4e). Substrate and chromaphore were added and colour development indicated the presence of a sample DNA sequence complementary to that of the immobilised primer.

The combination of results from wells containing the 2 different primers gave the Factor V genotype. If colour development was seen in only wells with primer 1 immobilised, then the individual had the normal Factor V. Colour development in both wells indicated that the individual was heterozygotic whereas colour development in only wells with primer 2 immobilised indicated a homozygotic Factor V_{leiden}.

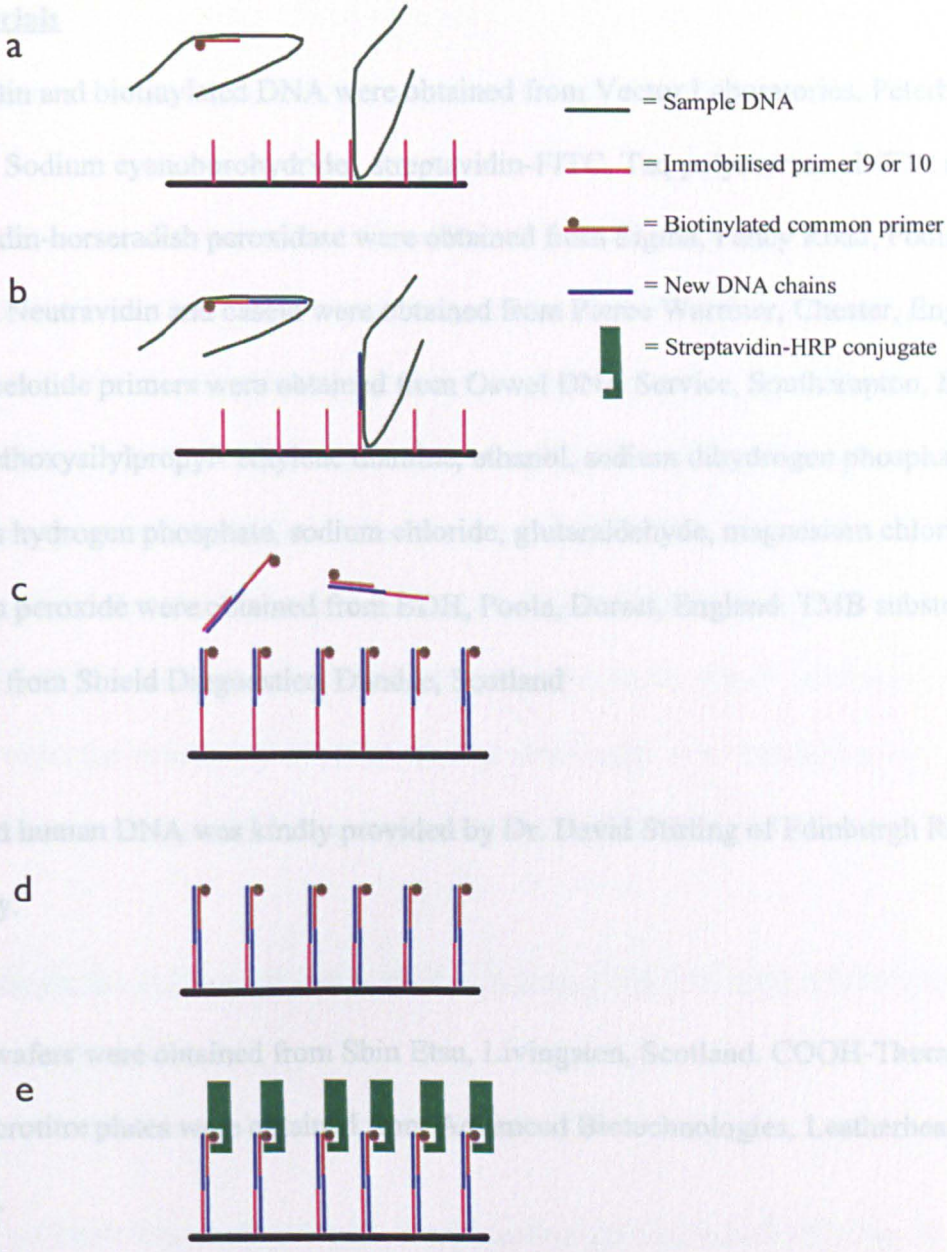


Figure 5.4 Principle of the assay for identification of Factor V genotype. a) Sample DNA binds to the immobilised specific Factor V genoytype primer if its sequences are complementary. Sample DNA also bind the biotinylated primer that is common to DNA for both genotypes. b) Elongation of primers occurs using sample DNA as template. c) With a new cycle primers bind to the newly synthesised DNA chains (as well as to original DNA) and amplification continues until d) biotinylated primers are attached to all of the immobilised primers via newly synthesised DNA chains. e). Streptavidin-HRP conjugate is added which attaches to biotin and allows colour development to occur thus demonstrating presence of genotype.

5.2 Materials

Photobiotin and biotinylated DNA were obtained from Vector Laboratories, Peterborough, England. Sodium cyanoborohydride, streptavidin-FITC, Taq polymerase, dNTPs and Streptavidin-horseradish peroxidase were obtained from Sigma, Fancy Road, Poole, Dorset, England. Neutravidin and casein were obtained from Pierce Warriner, Chester, England. Oligonucelotide primers were obtained from Oswel DNA Service, Southampton, England. 1,3-trimethoxysilylpropyl- ethylene diamine, ethanol, sodium dihydrogen phosphate, disodium hydrogen phosphate, sodium chloride, glutaraldehyde, magnesium chloride and hydrogen peroxide were obtained from BDH, Poole, Dorset, England. TMB substrate was obtained from Shield Diagnostics, Dundee, Scotland

Extracted human DNA was kindly provided by Dr. David Stirling of Edinburgh Royal Infirmary.

Silicon wafers were obtained from Shin Etsu, Livingston, Scotland. COOH-ThermoFast PCR microtitre plates were obtained from Advanced Biotechnologies, Leatherhead, England.

A Touchdown™ thermal cycler was provided by Life Sciences International, Basingstoke, England.

5.3 Methods

5.3.1 Patterning of nucleic acids using avidin and photobiotin.

Neutravidin was immobilised on a SiO_2 wafer using the silanisation process as described in Section 2.3.1. The avidin-modified wafer was incubated in 5 ml of 10 μg ml^{-1} long arm photobiotin in PBS for 20 minutes, and was then covered with 10 μg ml^{-1} biotinylated DNA and exposed to light as described in Section 2.3.1 through a mask with 10μm interval equal mark:space ratio lines. The wafer was covered with 10 mg ml^{-1} casein in PBS and exposed to light for 15 minutes in the absence of a mask. The wafer was then incubated in 10 μg ml^{-1} FITC labelled streptavidin in PBS, for 60 minutes. After washing in PBS and distilled water the sample was dried in a stream of nitrogen and examined using fluorescent microscopy. A photomicrograph of this surface is shown in Section 5.4.1.

5.3.2 Utilising the avidin-photobiotin immobilisation technique in the determination of Factor V genotypes

5.3.2.1 Immobilisation of oligonucleotide primers.

Wells of a COOH-ThermoFast™ plate were incubated with 1% (w/v) EDC in 10 mM phosphate buffer (pH 7.0) for 120 minutes at room temperature. The wells were then incubated in 200 μg ml^{-1} Neutravidin in 10 mM phosphate buffer (pH 7.0) for 16 hours at 4°C. All subsequent stages of the immobilisation process were performed at room temperature under dark room conditions. After washing in PBS, wells were incubated with 10 μg ml^{-1} long-arm photobiotin in PBS for 20 minutes. Solutions containing 1μg ml^{-1} of either primer 1 (normal sequence) or primer 2 (Factor V_{Leiden} sequence) were added to the wells and the plate was exposed to light from a high pressure mercury vapour lamp for 15

minutes. After washing in 10 mM phosphate buffer containing 1M NaCl and 0.1% Triton X-100 (to remove any non-covalently bound oligonucleotides) the plate was ready for use.

5.3.2.2 PCR amplification of DNA

Samples consisted of DNA extracted from the blood of individuals with known Factor V genotypes. 1 µl of sample was added to 50 µl of PCR reagent (20 U ml^{-1} Taq polymerase, 0.3mM dNTPs, 1.5 mM $MgCl_2$, 6 µg ml^{-1} biotinylated common primer in 10 mM Tris-HCl pH 7.6) in wells with both types of immobilised primer. The plate was then placed in the thermal cycler and PCR was performed using the following protocol:

Initial Denaturation step:		5 Minutes at 95°C
30 cycles of	Denaturation	15 seconds at 95°C
	Annealing	15 seconds at 55°C
	Elongation	30 seconds at 72°C
Final Elongation step		5 minutes at 72°C

5.3.2.3 Detection of PCR products

Following the PCR amplification, the wells were washed with PBS containing 1% BSA and 0.1% Triton X-100. A streptavidin-HRP conjugate was added to the wells and incubated at 20°C for 30 minutes, and wells were then washed with PBS containing 1% BSA and 0.1% Triton X-100 prior to the addition of the substrate solution (contained H_2O_2 and 3,3',5,5'-tetramethylbenzidine (TMB) as the chromophore). After 5 minutes incubation at 20°C, the addition of 1M H_2SO_4, stopped the action of the peroxidase and changed the colour of the chromagen from a pale blue to an intense yellow colour (λ_{Max} = 450 nm). Absorbances were recorded and are shown in Section 5.4.2 for people with different known Factor V genotypes (verified by PCR and gel electrophoresis).

5.4. Results and Discussion

5.4.1 Patterning of nucleic acids using avidin and photobiotin.

The photomicrograph of the surface shown in Figure 5.5 demonstrates that high resolution (<10μm) patterning of nucleic acids onto a surface could be achieved using the avidin-photobiotin technique developed in this work. It should be noted that the biotinylated DNA does not bind to the avidinated surface, via its inherent biotin groups. This is because the biotin binding sites of the immobilised avidin have been saturated by pre-incubation with photobiotin.

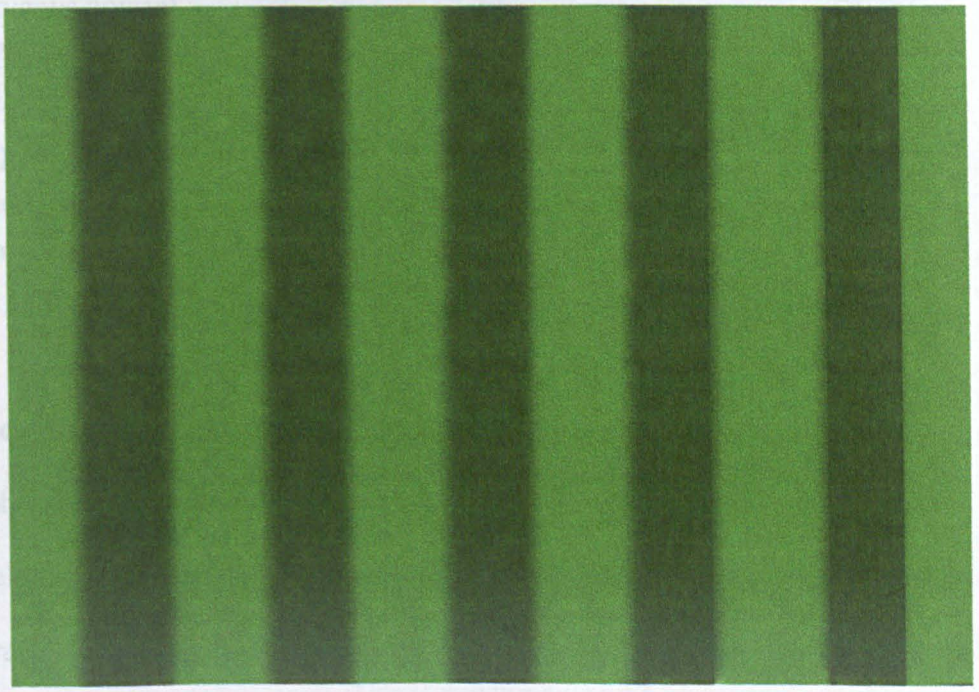


Figure 5.5 Photomicrograph the patterning of nucleic acids by use of the avidin-photobiotin technique. Avidin and photobiotin were immobilised on to the surface of a SiO2 wafer as described in Section 2.3.3. The surface was then covered with biotinylated DNA in PBS and exposed to light through a mask with 10 μm interval equal mark:space ratio lines. The wafer was covered with casein and exposed to light in the absence of a mask. The wafer was then incubated in FITC labelled streptavidin in PBS. Following washing the sample was dried in a stream of nitrogen and examined using fluorescent microscopy.

5.4.2 Utilising the avidin-photobiotin immobilisation technique in the determination of Factor V genotypes

The results shown in Table 5.1 indicates that by immobilising oligonucleotide primers with the avidin-photobiotin technique, and using this modified surface in conjunction with PCR and a suitable detection method it is possible to detect genetic polymorphisms. Negligible absorbances are obtained when the sample DNA sequence does not match that of the specific immobilised primer. Where sequences are complementary between sample and primer, high absorbances are obtained.

Sample	A_{450} Normal genotype primer	A_{450} Variant genotype primer
Homozygous normal	2.302	0.067
Heterozygous	1.892	1.726
Homozygous variant	0.078	2.251

Table 5.1 Results obtained from patients with different Factor V genotypes. Individuals with a homozygous normal genotype only show a reaction with the oligonucleotide primer for the normal genotype. Individuals with a homozygous variant genotype only show a reaction with the oligonucleotide primer for the variant genotype, whilst individuals who are heterozygous show reactions with both primers.

It may be possible to construct sensor arrays for use in genetic analysis using the avidin-photobiotin immobilisation technique to selectively immobilise different oligonucleotide primers to specific parts of the array. The substrate of the array could incorporate an element allowing its temperature to be cycled, and thus an integrated device could be fabricated that would have primers capable of recognising specific nucleotide sequences and the capability of obtaining the temperature control necessary for PCR without the requirement for expensive thermal cyclers. A major problem in the construction of a device that incorporates the DNA amplification stage would be the elimination of non-specific binding. Because of the tremendous amplification achieved by PCR, even the presence of one molecule of non-specifically bound nucleotide sequence could have a seriously detrimental effect.

5.5 Review of related work from other groups since publication of the avidin-photobiotin immobilisation technique.

Since the first description of using photobiotin to selectively immobilise biological molecules[197], a number of other workers have used the technique or modifications thereof.[311-317]

The avidin-photobiotin patterning method has been applied to direct sensing detection systems.[311,312] Figure 5.6 shows a SPR microscope image of IgG patterned on to a surface using the avidin-photobiotin method.[311] The pattern was created using a a mask with 50 μm wide equal mark:space ratio lines.

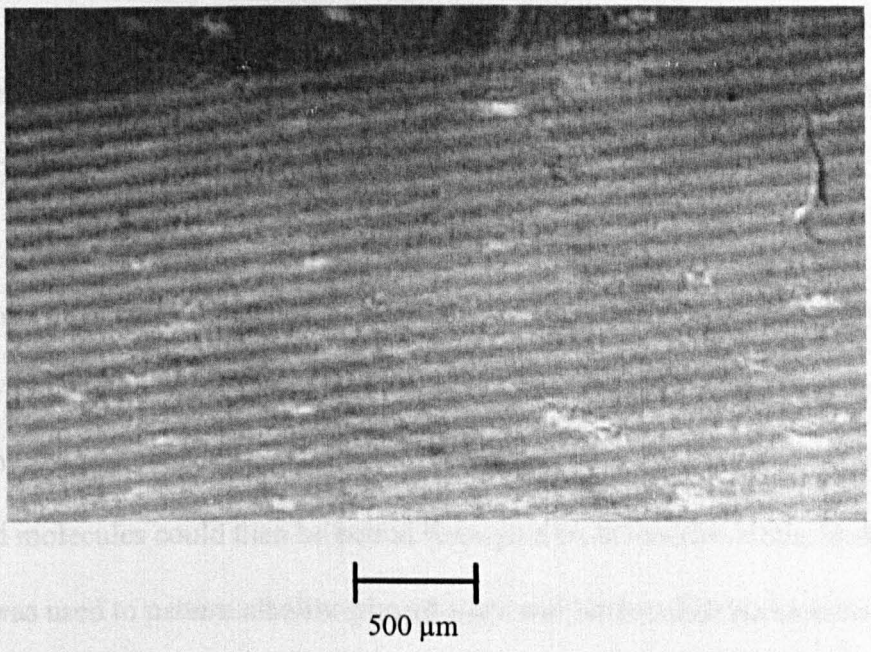


Figure 5.6 Surface plasmon resonance microscopy image of IgG patterned on to a surface using the avidin-photobiotin method. The pattern was created using a a mask with 50 μm wide equal mark:space ratio lines.[311]

One group used avidin/photobiotin to attach enzymes to a carbon electrode surface such that the enzymes were spatially separated and directly adjacent to electrode transfer sites on the same electrode surface.[313] High resolution patterning of the enzymes was required, so that the distance between the enzyme-loaded domains and the electron transfer sites was less than 5 μm, so as to maintain the fast response time and high sensitivity required for the measurement of neurotransmitter dynamics. Patterning of enzymes was achieved by use of an interference pattern generated from a UV laser. Characterisation of the surfaces was performed using fluorescence microscopy and AFM. This group has also shown that extensive biotin/avidin derivatisation of carbon electrode surfaces results in a decrease of the electron transfer rate of the electrode, but use of photobiotin to specifically immobilise proteins directly adjacent to electrode transfer sites (where photobiotin has not been activated, and protein immobilisation has not occurred) on the same electrode surface maintained fast response times and high sensitivity.[314] The use of an interference pattern generated from a UV laser to activate photobiotin has recently been used to immobilise antibodies onto a carbon surface with with sub-micron resolution.[315]

Other workers have created patterns of biotin on polymer (polystyrene and nitrocellulose) surfaces, by exposing the polymeric surface to photobiotin in the presence of light through a mask.[316] The subsequent addition of avidin resulted in the formation of surfaces to which biotinylated molecules could then be bound through a biotin-avidin-biotin bridge. The technique was used to pattern alkaline phosphatase and horseradish peroxidase whilst retaining their catalytic activity. It should be noted that this method is very similar to that described in Sections 2.3.1 and 2.13.1, the difference being the use of a polymer instead of a protein modified surface as the matrix for attachment of photobiotin.

A variation on the photobiotin based immobilisation method, is the use of a "caged" biotin analogue.[317] The biotin analogue (which contains a photoactivable nitroveratryloxy-carbonyl (Nvoc) group) is covalently linked to a substrate surface. The Nvoc group blocks the avidin-biotin interaction, but upon exposure to UV light it is cleaved yielding biotin sites which can be used to immobilise biotinylated macromolecules via a biotin-avidin-biotin bridge. Patterning can be achieved by the use of a photolithographic mask.

Advances have been made in other methods of patterning biological molecules, particularly with substrates containing micron and sub-micron scale channels.[318,319] The availability of micro machined dispensing systems capable of dispensing picolitre quantities of liquids,[320,321] provides the opportunity of physical deposition of material with high resolution. Sub-micron resolution patterning of immunoglobulins has been achieved using microfluidic networks, using gold, glass and polystyrene as substrates.[318]

5.6 Future possibilities

The work presented in this thesis has demonstrated the development of a novel method for selectively immobilising biological molecules in predefined areas with micron scale resolution, and its use in a number of applications. There are a number of areas in which the work outlined in this thesis could be taken further.

5.6.1 Multianalyte Enzymic biosensors

The successful fabrication of a multianalyte enzymic biosensor using ultramicroelectrodes was demonstrated in Chapter 4. Future work could be undertaken to construct arrays of electrodes measuring a large number of analytes simultaneously. In these arrays a number of sensor elements could measure the same analyte in parallel, which would allow more accurate and reproducible determinations (through averaging) and minimising the effect of (or even excluding) any sensor elements producing a result considered to be an outlier. The avidin-photobiotin immobilisation technique could also be applied to the manufacture of multianalyte optical biosensors. One particularly attractive feature of this application is that for sensors such as those employing fibre-optic technology the light necessary for immobilisation could be delivered by the sensors tranducing element itself, which would make fabrication very simple.

5.6.2 Multianalyte Immunosensors

The successful fabrication of a number of multianalyte immunosensors has been demonstrated in the course of this work. In Chapter 2 the fabrication of qualitative immunosensors (both optical and amperometric) for up to 5 analytes was demonstrated. In Chapter 3 a quantitative amperometric multianalyte immunosensor for the determination of gonadotrophins was constructed, and in Chapter 4 the feasibility of using very small electrodes in multianalyte amperometic immunosensors was shown.

Arrays of electrodes could be constructed, and as for enzymic sensors (Section 5.7.1) these could be used in controlling the quality of the results produced. Again as for enzymic systems the avidin-photobiotin method could be utilised in the production of optical immunosensors.

5.6.3 Integrated devices for analysis of nucleic acids.

There are numerous reports in the literature of biosensors designed for the analysis of nucleic acids.[322-324] One potential application of an immobilisation technique such as the one developed in the course of this work, is in the fabrication of an integrated device for the amplification and analysis of nucleic acids. As discussed in section 5.4.4, oligonucleotide primers complementary to a variety of nucleic acid sequences could be immobilised onto elements of an array on a substrate that incorporates an element capable of controlling its temperature. This sensor could be placed in contact with the sample and reagents and its temperature cycled to perform the PCR amplification.

A multianalyte sensor constructed in this way could be very small, and thus very rapid temperature changes could take place resulting in a shortening of assay time. A number of potential detection methods exist including fluorescence, amperometry or direct methods such as SPR. As was discussed in section 5.4.4. a likely major problem in the construction and use of such a device would be the complete elimination of non-specific binding (due to the ability of the PCR reaction to amplify minute amounts of nucleic acids).

5.6.4 Direct sensing

The avidin-photobiotin immobilisation method could be used for multianalyte direct and real-time sensing of reactions involving a change of mass at the sensor surface. Such reactions would include immunological reactions and detection of nucleic acid amplification products as discussed in section 5.6.3. The combination of direct sensing techniques with the avidin-photobiotin immobilisation procedure has already been demonstrated (Section 5.5).[311,312]

5.6.5. Cell Guidance

A further possible application of this immobilisation procedure is in the fabrication of a device for the directional propagation of an individual cell, (e.g. nerve cell processes) on a patterned substrate. In this case, the essential prerequisites are the same as for the design of a diagnostic device, in so much that it is desirable to position molecules as a pre-defined pattern on a substrate with no interference from non-specific adsorption. Patterned molecules may act as a chemotactic or topological template for the guidance of the cell, which grow preferentially in a given direction determined by the pattern.[142] Particularly important applications would be in the manufacture of devices either to control endothelial cell growth for wound healing, or to control nerve cell growth to promote regeneration.

In addition to the design of devices for the guidance of individual cells, it may be desirable to use a patterning technique to alter the behaviour of many cells. For example, by coating appropriate molecules onto a surface, it may be possible to differentially promote or prevent

cell adherence and growth on the outer surface of a miniature implantable *in vivo* sensor, in order to enhance the biocompatibility properties of the device.

An initial investigation was performed to assess if the avidin-photobiotin immobilisation technique could be used for guiding cell growth. Briefly, concanavalin A and BSA were patterned on to a silicon dioxide wafer. Snail neurones were placed on the substrate and incubated in growth media for 7 days. The cells were examined under a microscope. No guidance of the cell processes was evident, whereas in a control where protein had been patterned using a photolithographic lift-off technique[142] (see section 1.9.2) guidance of cell processes was evident with cellular processes running parallel to each other with distances between them reflecting the periodicity of lines on the photolithographic mask. The effectiveness of both patterning techniques was validated by patterning of biotinylated Concanavalin A, followed by incubation with a streptavidin-FITC conjugate. The reason for the difference in cell guidance between surfaces patterned with protein by these two techniques is not known. The lift-off technique may provide a firmer topological guide to cells than does the avidin-photobiotin technique, or changes to deposited protein as a result of the exposure to organic solvents in the lift-off technique may be influential if cell guidance is chemotactic in nature.

5.6.6 Other applications of patterning technique

The immobilisation procedure developed in the course of this work could also be used to selectively deposit molecules onto a surface in ordered arrays for applications other than diagnostic sensors. Using this procedure, groups of molecules (which may possess particular properties, for example photodynamic proteins) may be positioned precisely with respect to other groups of molecules, or to other structures in order to build up functional molecular architectures. As in the case for the design of a diagnostic device, a variety of different transducer materials may be used as immobilisation substrates.

5.6.7 Photoactivable Reagents other than Aryl Azides

There are a number of reactive groups other than aryl nitrenes which may be formed photochemically. Carbenes may be formed from diazo compounds and diazirines, and are even more highly reactive than aryl nitrenes.[204] Nitrophenyl ethers can undergo photochemical aromatic substitution selectively at amino groups, and psoralens have been used to react with pyrimidine and purine groups in nucleic acids. Other photactivable groups include non aryl azides, α,β-unsaturated ketones, aryl halides, nitroaryl compounds, purines and pyrimidines.[204] A major advantage of using photactivable reagents other than aryl azides would be in orientation of the immobilised molecule. A particular example where this would be extremely useful would be the patterning of oligonucleotides. Although the immobilisation technique employing an aryl azide (in the photobiotin molecule) was successful in that DNA amplification and detection occurred, it is likely that a significant proportion of the immobilised oligonucleotide was unable to act as a primer due to steric hinderance. As aryl azides react with a very wide range of species, the

photobiotin may attach the oligonucleotide such that subsequent binding of nucleotides or the action of Taq polymerase are inhibited. Oligonucleotides with an amino group at one end are easily synthesised and are commercially available. Selective immobilisation using a photoactivable reagent such as nitrophenyl ethers that react selectively with amino groups would allow optimal orientation of the nucleotide for DNA amplification and detection. Photoreactive compounds containing specific peptide sequences promoting cell adhesion have been synthesised, and have been used to prepare 2-dimensional cellular patterns.[325]

5.6.8 Very High Resolution Patterning

The resolution of the protein pattern that can be "written" using a photolithographic mask is limited to approximately half the wavelength of light used.[326] Other workers have demonstrated sub-micron resolution when employing avidin-photobiotin, by use of laser interferometric techniques (Section 5.5).[315] Another potential approach to patterning of biological molecules with improved resolution is by the use of a near-field optical scanning microscope (NFOSM).[327,328] This instrument is capable of achieving resolution of about 20 nm. In a NFOSM a tiny aperture, typically 10 nm in diameter, is positioned at the apex of a conical metal screen. This aperture is illuminated from its reverse side and is scanned over the sample in very close proximity to it, maintaining the sample tip gap by utilisation of the tunnelling current as in STM. This technique has been applied to the generation of submicron scale patterns in photoresists,[329-331] and it is possible that it could be applied to the avidin-photobiotin patterning technique.

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He ranged his tropes, and preached up patience;

Backed his opinion with quotations.

Matthew Prior

Paulo Purganti and his wife

You will find it very good practice always to verify your references, Sir!

Martin Joseph Routh

John William Burgon Lives of Twelve Good Men

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