SULFAMETHAZINE IN FOOD: A NEW APPROACH TO SCREENING

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ABSTRACT

The aim of the research was to use the principles of immunoassays and fluorescence spectroscopy to detect the presence of 4-amino-N-(4,5-dimethyl-2-pyrimidinyl)benzenesulfonamide **(1)**, commonly known as sulfamethazine, in food.

The majority of the work was centred on the method of detection specifically on the synthesis of a fluorescently labelled sulfamethazine conjugate. It was necessary to use labelling reagents that did not disrupt the antibody binding affinity of the sulfamethazine or the fluorescence characteristics of the label.[1,2,3]

Fluorescein isothiocyanate **(34)** was initially used to label sulfamethazine and subsequently several symmetrical sulphoindocyanine dyes, **(35)**, **(36)** and **(51)**, were synthesised. These were reacted with sulfamethazine to give conjugates the **(52)**, **(57)**, **(60)** and **(61)** respectively. It was possible to observe the fluorescence of conjugates **(52)** and **(60)** in both a liquid phase and on a solid support, which allowed them to be used in the production of a competitive fluoroimmunoassay.

In the development of the competitive fluoroimmunoassay polyclonal antibodies and a blocking agent were immobilised on to a polymer membrane. The tailoring of the antibody concentration and blocking conditions allowed the fabrication of a crude test strip. Sulfamethazine from the sample was allowed to bind to the antibodies on the test strip, and the fluorescent conjugate competed for the remaining antibody binding sites. The measurements of the bound fluorophore conjugate in the absence of sulfamethazine resulted in a high fluorescence signal, which decreased when sulfamethazine was present. Therefore the signal detected from the fluorescent conjugate was inversely related to the concentration of sulfamethazine in the sample.

Optimisation of the immunoassay conditions resulted in a qualitative test strip with a sensitivity of 10 μg/l and a 26 minutes assay time. This was suitable for sulfamethazine screening.

ABBREVIATIONS

Φ	Quantum Efficiency
ε	Molar Absorbance Coefficient
λ_{max}	Maximum Excitation Wavelength
Ab	Antibody
Competitive Assay	Used to describe an antibody limited assay, although no direct competition between antigen and tracer occurs
Cy5.18.OH	650 nm pentamethine cyanine dye **(35)**
Cy5.18.OSucc	Succinimidyl ester of Cy5.18.OH **(47)**
Cy7.18.OH	765 nm heptamethine cyanine dye **(36)**
Cy7.18.Osucc	Succinimidyl ester of Cy7.18.OH **(48)**
DMF	N,N-Dimethylformamide
DMSO	Dimethylsulfoxide
e^-	Used throughout this report as x 10^-
EDC	1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride
FITC	Fluorescein isothiocyanate **(34)**
HMPT	Hexamethylphosphoric triamide
HRP	Horseradish Peroxidase
HRP-SMT	Horseradish peroxidase – sulfamethazine conjugate
MeOD	Deuterated Methanol
MRL	Maximum Residue Limit
NC	Nitrocellulose
PBS	Phosphate buffered saline
Plates	Polystyrene 96 well microtitre plates
PVA	Polyvinyl alcohol
PVDF	Polyvinylidene fluoride
SMT	Sulfamethazine **(1)**
THF	Tetrahydrofuran
TMB	3,3',5,5'-Tetramethylbenzadine Chromogenic Substrate

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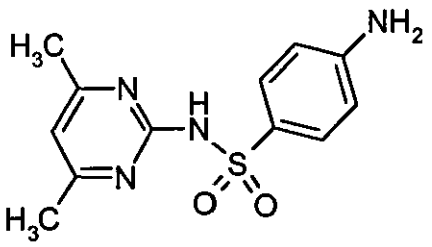
CHAPTER ONE

INTRODUCTION

1.1 Aims of the Project

The aim of this research was to use the principles of immunoassays and fluorescence spectroscopy to detect the presence of 4-amino-N-(4,5-dimethyl-2-pyrimidinyl)benzenesulfonamide **(1)**, commonly known as sulfamethazine, in food.



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Currently, the most common method used for the detection of sulfamethazine in food samples is by an enzyme-linked immunosorbent assay (ELISA). These assays have the major drawback in that they commonly use microtitre plates, which are both labour intensive and time consuming.[4] It was anticipated that the development of a fluoroimmunoassay would provide a relatively simple and inexpensive screening procedure for the qualitative detection of sulfamethazine in food.

The majority of the work has centred on the method of detection. We have synthesised fluorescently labelled sulfamethazine conjugates and used these to develop an immunoassay on a test strip. The conjugates were made using 490 nm (visible), and 650 nm and 790 nm (near infrared) dye fluorophores. It was possible to observe the fluorescence of these fluorophore-sulfamethazine conjugates in solution and when adsorbed onto a solid support, which made it possible to produce a fluoroimmunoassay. We have tailored the immunoassay conditions to give a rapid, highly sensitive, competitive fluoroimmunoassay which is suitable for sulfamethazine screening.

1.2 Sulfamethazine

Sulfamethazine is a member of a group of broad-spectrum antibiotic compounds known as the sulfonamides*. Sulfonamides have been prescribed for use in humans and animals since the early 1940's. They continue to be popular because of their wide range of activity and economic advantages due to an inexpensive production. Sulfamethazine has been proven to be effective for maintaining the health of farm animals and is most often used to treat bacterial and protozoan infections. It works by inhibiting the enzymes involved in the synthesis of folic acid, which a number of organisms use as essential growth factors.[5] The action of sulfamethazine is therefore bacteriostatic. Successful treatment depends on an adequate concentration being maintained in the body for a long enough period of time to allow the animals' own defence mechanisms to destroy the pathogen.[6] It is routinely used in food-producing animals such as pigs, cattle, poultry and farmed fish in both a therapeutic and prophylactic capacity, and when used in sub-therapeutic concentrations, sulfamethazine exhibits a growth promoting action.[7,8]

To minimise the risk of sulfamethazine and other antimicrobial residues being present in the human diet by the consumption of meat, the substances are withdrawn from the animals' food several weeks prior to slaughter. However, withdrawal periods for antimicrobial agents vary with type of animal, routes of administration, active ingredients, and manufacturer. A few therapeutic preparations have withdrawal periods of less than 5 days, but the majority are between 5 and 30 days.[9,10] In fish and other poikilotherms withdrawal problems arise due to the slower excretion rate when compared with endothermic animals. Consequently, high residue levels will be present in the meat if the exact withdrawal periods are not strictly observed.[11]

The presence of drug residues in food may cause a health problem for the consumer. The potential toxic effects in humans include renal damage, thyroid damage and allergic reactions. This coupled with the report that

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* Sulfonamides are compounds which contain the RSO_2NR_2 structure

sulfamethazine may be a carcinogen, gives concern over the presence of these residues in meat and milk.[12,13,14] Moreover, several reports in 1988 and 1989 showed sulfamethazine to be present in market milk and imported pork.[15,16] It is for these reasons that the use of antibiotics in livestock is tightly controlled.[17]

1.2.1 Residue Monitoring

In order to have control over the use of antibiotics in food producing animals, the European Union limits the type of veterinary drugs, monitors their use and operates a random screening programme. Each permitted veterinary drug has been assigned a maximum residue limit (MRL), Appendix 1.[7] The MRL is the maximum concentration of residue resulting from a veterinary medicine that is legally permitted, or recognised as acceptable, in food. These set limits are considered to be without any toxicological or microbiological hazard, and are an estimate of the amount of a veterinary drug that can be ingested daily, over a lifetime, without appreciable health risk. In 1990, a preliminary MRL for sulfonamides was set as 100 μg/kg (100 ppb), and this became the recognised acceptable limit for use in milk, muscle, liver and kidney in 1994.[7,18] It became a prosecutable offence to sell any goods that contain sulfonamide residues above this legal limit. Residue screening was recommended throughout Europe but, as each country has its own methods of antibiotic testing, no standard screening procedure has been implemented.

The European Union demands that two independent analytical procedures be used in order to secure a prosecution for illegal sulfamethazine use. With this situation in mind, most of the analytical procedures that were previously employed as screening methods are now being used as the final confirmation technique.

In the United Kingdom the Veterinary Medicines Directorate (VMD) operates two complementary surveillance programmes. The statutory surveillance programme fulfils the UK's residue screening obligations, Directive 86/469/EEC, and the supplementary non-statutory programme extends the analyte and matrix range to include combinations not covered

by the statutory test.[19] Both of these programmes play a central role in ensuring that the consumer is protected against potentially harmful veterinary medicine residues. Each year the State Veterinary and Meat Hygiene Services collect over 40,000 random samples from selected farms and slaughterhouses and pass these on to the Veterinary Laboratories Agency, which carries out analyses on kidney samples to test for antimicrobials. Total antimicrobial screening is carried out by a microbiological procedure but this does not identify the samples containing low concentrations of sulfonamides. Therefore, a separate sulfonamide analysis is carried out by high performance thin layer chromatography (TLC) followed by confirmation by high-pressure liquid chromatography (HPLC).

In 1996, the statutory surveillance survey revealed that 0.70% of pigs contained antimicrobial compounds, of these 1.7% were found to contain sulfonamides. It is safe to say that the majority of samples analysed for veterinary drug residues are negative. Consequently a demand for an economical method of residue screening that can rapidly identify such samples has arisen, this should allow more resources to be concentrated on identification and quantification of the few positive samples.[20]

1.3 Methods of Sulfamethazine Analysis

Presently the determination of sulfamethazine is based on qualitative and quantitative methods of analysis. Qualitative methods, such as TLC, microbiological inhibition assays, and immunological analysis are widely used.[21,22,23] The microbial techniques are simple but their sensitivity and selectivity are insufficient.[24] Instrumental analytical methods such as HPLC and gas chromatography coupled with mass spectroscopy (GC-MS) are used for the quantitative determination of sulfamethazine.[25,26] Although accurate and sensitive, the instrumental methods are expensive, time consuming and often require elaborate sample clean-up procedures.

1.3.1 Immunoassays for Sulfamethazine

The demand for cheap, high throughput analytical systems for measuring drug residues in food led to the development of sulfamethazine immunoassays. Yalow and Berson reported the first immunoassay in 1959 with their radioimmunoassay for the detection of insulin.[27] However, this new technology was slow to take off within the food industry. The first food radioimmunoassay was reported at the International Biological Programme in Stockholm in 1968.[28] By 1973, the number of immunoassays used in food testing had risen to only seven. When an enzyme immunoassay was developed the use of immunoassays in food analysis expanded exponentially. By 1984 the total number of food immunoassays represented 45% of the total food analyses, the remaining 55% of tests were performed using other traditional methods.

Many immunoassays for the determination of sulfamethazine are already in existence, most being based on enzyme-linked antigens. The determination of sulfamethazine using a horseradish peroxidase labelled antigen (HRP-SMT) is very popular and has been used to determine sulfamethazine mainly in microtitre plate and, more recently, on solid surface membranes.[75,33] Ostermaier *et al.* have recently developed a system in which several sulfonamides can be determined simultaneously on a solid matrix test strip using HRP-SMT.[29] Jackman *et al.* developed a robust enzyme immunoassay with a 20 minute incubation time. The assay involves direct competition of the antigen and HRP-SMT for the antibody, and the addition of a chromogenic horseradish peroxidase substrate. This gives a blue coloured product, whose intensity at the end point is inversely proportional to the concentration of sulfamethazine.

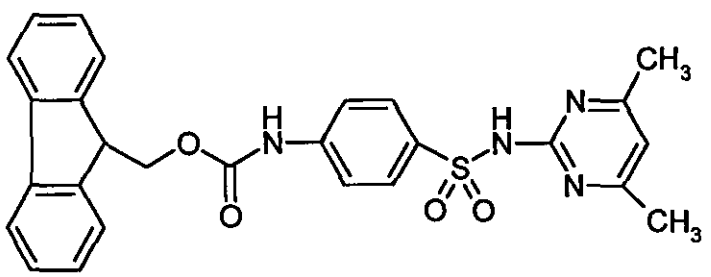
The enzyme immunoassays rely on microtitre plates and although the assays are quite quick, the preparation of microtitre plates, enzyme substrates and plate washing solutions are time consuming. They have the disadvantage of not being directly comparable. All the plates are unique and although comparisons can be made throughout a plate, plate-to-plate variations can occur.

Recently several rapid sulfamethazine enzyme linked immunosorbent assay (ELISA) kits have become commercially available from IDEXX, Rhône-Poulenc and SmithKline Beecham.[30,31,32] These kits show the desired sensitivity and selectivity and do not need complicated extraction and clean-up steps.[33] The immunochemical results are comparable to quantitative high performance TLC and HPLC with electrochemical detection, having detectabilities in the low parts per billion ranges.[34]

1.3.2 High Pressure Liquid Chromatography (HPLC)

HPLC is the most widely used technique for sulfonamide determination.[35] It has excellent sensitivity and can easily detect sulfamethazine below the 100 μg/l MRL. However, HPLC analysis usually requires sample pre-treatment in order to remove interfering compounds and then UV detection.[36]

Several methods have been reported which involve the formation of a fluorescent derivative of sulfamethazine to increase the assay sensitivity.[37,38] The work of Cross *et al.* involved a 9-fluorenyl-methyl-chloroformate to give derivative **(2)**, separation by HPLC, and UV detection.[39] Takeda and Akiyama reported the determination of eight sulfa drugs by pre-column derivatisation using fluorescamine, and Stringham *et al.* used a post-column sulfamethazine derivatisation with dimethylaminobenzaldehyde and further HPLC separation.[40,41]



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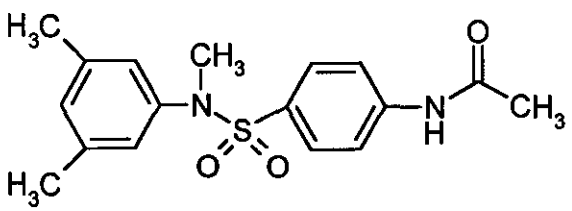
There are HPLC analyses that do not involve sulfamethazine derivatisation. However, they require extra filtration and extraction steps,

which include the use of chloroform, hydrochloric acid and buffers, further separation by HPLC or C-18 reverse-phase liquid chromatography, followed UV detection at 280 nm.[42,43]

1.3.3 Gas Chromatography (GC)

GC methods are not as widely accepted as HPLC, yet the sensitivity and specificity are comparable. GC requires solvent extraction, sample clean up, and derivatisation before analysis. It is necessary to convert sulfamethazine into a suitable derivative that is highly electronegative and amenable to sensitive electron-capture detection.[44] The most common derivative is **(3)**.[45,46,47,48]



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1.3.4 GC-MS and LC-MS

The high cost of the specialist equipment needed for GC-MS and LC-MS has severely limited their use for analyte screening. However, due to their high selectivity, sensitivity, and specificity they are used as confirmatory methods.[49,50,51]

Matusik *et al.* have described a method of GC screening followed by GC-CI^+MS confirmation for the analysis of sulfamethazine and two of its metabolites, desaminosulfamethazine and N^4-acetylsulfamethazine.[52] Weber and Smedley[53] described the screening of milk by LC followed by GC-MS confirmation, and Carignan *et al.*[54] described a method that employs an LC sample purification followed by GC-MS analysis.

1.3.5 Other Methods of Detection

TLC is generally used for rapid analyte screening, in particular, sulfonamide drug residues present in food. Unfortunately this method still requires sulfamethazine derivatisation.[55,56] Other less well known methods for sulfamethazine detection include photochemically induced fluorescence, colorimetric determination, electrochemical detection and fluorescence polarisation.[57,58,59,60] Jackman *et al.* described a polarisation fluoroimmunoassay using an automated analyser, which is capable of detecting approximately one sample per minute.[1]

1.3.6 Methods of Extraction

All the methods of detection require the sulfamethazine to be extracted from the sample. Several methods have been developed for this purpose. The Tishler extraction and clean-up method involves steps that use chloroform-acetone followed by hexane-acetone extractions. Extraction with 1N HCl, wash with alkali, and a final extraction with chloroform before complexing with N-(1-naphyl)-ethylenediamine hydrochloride and GC analysis.[61]

For simpler HPLC detection procedures, sulfamethazine from the sample is extracted into an organic solvent such as chloroform or methanol. The sample is further extracted into buffers then passed through a solid phase extraction column before being analysed.[62,63]

Supercritical fluid extraction (SFE) has been coupled to mass spectroscopy and ELISA for use as detection techniques.[64] SFE has the advantage over other extraction methods because the amount of sample and solvent used are minimal.[65] Super-critical extraction using CO_2 has shown to give poor recoveries of sulfamethazine, however this has been overcome by using super-critical fluoroform, subcritical freon134A or solvent modified super-critical CO_2.[66,67,68] Modification of super-critical CO_2 using acetonitrile gave 90% recovery of sulfamethazine when extracted from chicken liver.

Schwartz and Lightfield described a relatively simple and inexpensive procedure for screening milk for sulfamethazine.[69] Milk is passed over Chromosorb 102, which absorbs sulfamethazine, the drug is eluted, and purified by passing the effluent over small beads of buffered anion-exchange resins and alumina. The sulfamethazine is finally isolated and analysed.

The simplest sulfamethazine extraction has been reported by Patel *et al.*[33] Their method involved homogenisation of a kidney sample with a buffer, centrifugation, followed by removal of the supernatant which is used directly in an immunoassay.

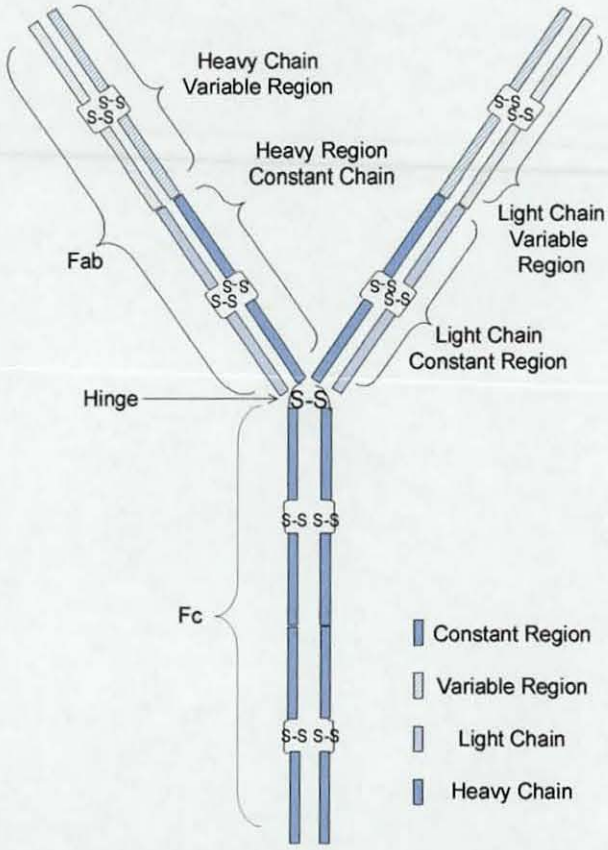
1.4 Immunoassays

An immunoassay is an analytical procedure that uses antibodies for the qualitative or quantitative detection of a specific antigen (or analyte)[†]. These techniques are particularly suited to the detection of low concentrations of antigens in biological matrices, due their high selectivity, sensitivity and versatility.[70] They take advantage of the molecular recognition, high selectivity and affinity of the antibody for the antigen.[71]

Antibodies are glycoproteins which belonging to the immunoglobulin family. They are produced *in-vivo* by fully differentiated B-cells within the plasma in response to a foreign substance, or immunogen, as part of the body's defence mechanism. Antibodies comprise mainly of one group of immunoglobulins, immunoglobulin G (IgG), which have an approximate molecular weight of 150,000 Daltons. All antibodies have the same basic structure. When visualised under an electron microscope they can be seen to be Y shaped molecules whose arms can swing out to an angle of 180°. They consist of four polypeptide chains, two identical heavy chains and two identical light chains which are held together by disulfide bonds (Figure 1.1).

[†] The term's analyte and antigen are interchangeable.

Figure 1.1 Representation of the Basic Structure of IgG



Initial studies based on amino acid sequencing demonstrated that the heavy chain and light chains consist of two types of regions, the variable region and the constant region. The variable regions are complementarity-determining and form the antigen binding sites and govern the specificity. These are known as the antibody binding fragments (F_{ab}). The constant region is used in recognition of the antibody by the host cells and is used during antibody replication. This region plays no part in the antigen binding and is termed the crystalisable fragment (F_c region).[72]

Small antigens, e.g. drugs, may not be immunogenic and are referred to as haptens. Haptens require conjugation to carrier proteins to induce an antibody response. The site and means by which the hapten is linked to the carrier protein determines the degree of specificity and quality of the antibody.[73] An adjuvant is co-administered with the immunogen in order to ensure slow release of antigen from injection sites. This has the effect of stimulating the immune system thereby enhancing the responses to the administered antigen.[74]

1.4.1 Antigen-Antibody Binding

The binding of an antigen to an antibody is a non-covalent, reversible, bimolecular interaction involving four types of intermolecular forces.[74] These are electrostatic, hydrogen bonding, hydrophobic and Van der Waal forces.[71,72] It has been estimated that hydrophobic interactions may contribute up to 50% of the total strength of the antigen-antibody (Ag-Ab) bond.[75] The one essential feature in all four forces is the close approach of both molecules before the forces become significant. This is at the heart of the combination of antigens to antibodies. By having complementary electron clouds on the antibody and the surface determinant of the antigen, the two molecules can fit snugly together. The greater the area of antigen and antibody fit the greater the forces of attraction, particularly if opposite charges and hydrophobic groups are present.

1.4.2 Antibody-Antigen Interactions

The reaction between an antigen and antibody is diffusion controlled (Equation 1.1), and at equilibrium obeys the Law of Mass Action:‡

$$\underset{\text{(Free fraction)}}{Ag + Ab} \underset{k_d}{\overset{k_a}{\rightleftharpoons}} \underset{\text{(Bound fraction)}}{Ag\text{-}Ab} \qquad \text{Equation 1.1}$$

Ag represents the antigen, Ab the antibody, and Ag-Ab the bound complex.

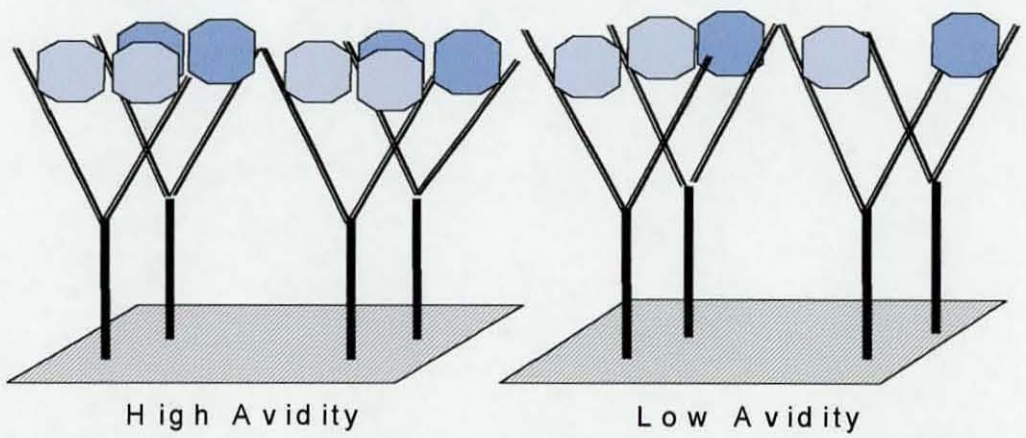
At equilibrium the free reactants will be combining, with a rate constant of k_a to form more of the complex, whilst the complex will be dissociating with the rate constant k_d to give free antigen and antibody.[76] The affinity constant k_a for polyclonal antibody reactions is within the range:[74]

$$10^4\ \text{lmol}^{-1} > k_a > 10^{12}\ \text{lmol}^{-1}$$

‡ **Law of Mass Action**: the principle that the rate of a chemical reaction is proportional to the masses of the reacting substances.

Antibody affinity has its most precise application when a monovalent F_{ab} fragment from a monoclonal antibody reacts with a monovalent antigen. However when intact antibodies are used, the possible multivalency of the antigen and the contribution of secondary interactions do not allow precise affinity measurements. This is most evident when polyclonal antibodies react with a complex antigen expressing mulitple non-identical epitopes. The ability of an intact antibody or polyclonal antiserum to bind its antigen is evaluated in terms of its avidity.[175] Although based on affinity, the avidity is recognised as the summation of all of the individual interactions that are taking place under a given set of conditions employed within an assay.[§] It is a measure of the overall stability of the Ag-Ab complex and reflects both the antibody valency and affinity (Figure 1.2). In general, high avidity antibodies give faster, more sensitive immunoassay.

Figure 1.2 Representation of Antibody Avidity



In solid-phase immunoassay techniques, external diffusion limitations play a role in the analysis. Sadana and Chen indicate that surface diffusion-controlled reactions can occur in clusters or islands and exhibit anomalous kinetics.[77,78] These fractal kinetics give unusual reaction orders and time-dependent rate coefficients.

[§] It is common to find such determinations reported in the literature as measurements of affinity; whilst strictly incorrect this practice is very widely followed.[175]

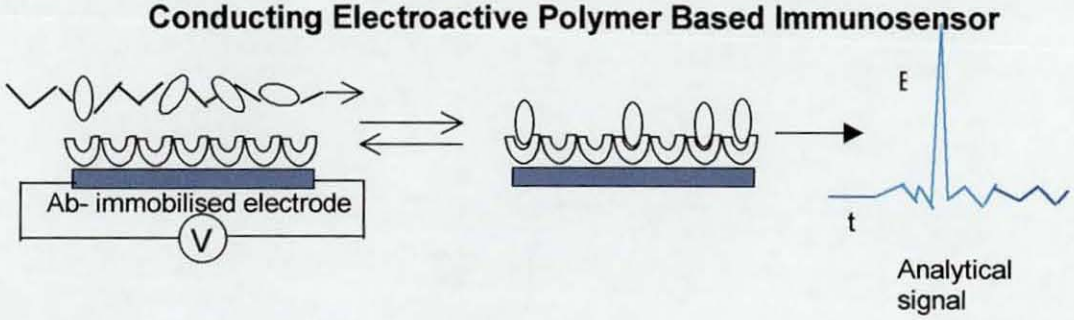
1.4.3 Detection of Immunological Reaction

The complex formed when an antigen and antibody react cannot be directly distinguished from free antigen and antibodies; consequently, a method of detection is required. Numerous analytical methods have been developed to generate and amplify analytical signals resulting from Ag-Ab interactions; many involve the labelling of the antibody or antigen.

Immunoassays do not always require the use of labelled reactants for example, monitoring the precipitation line that forms in a gel support when a protein antigen comes into contact with its specific antibody.[79] This technique is limited to the assay of proteins and other large molecules present in high concentrations and has the disadvantage of needing large amounts of mono-specific antisera.[76]

Several researchers have reported direct monitoring of immunological reaction by observing changes in electrical or optical properties due to Ag-Ab binding.[80,81,82] The advantages of these techniques include rapid detection times with real-time monitoring possibilities, no separation requirements and minimal use of reagents.[83] However, difficulties have been encountered in fabrication of these immunosensors due to problematic antibody immobilisation onto the transducer or fibre-optic and efficient generation of analytical signals.[84] Efforts are now being directed to the development of new conducting electroactive polymers and membranes for antibody immobilisation, such as polypyrrole, polyaniline and polythiophene.[85,86] The antibody interaction with the antigen is controlled through the application of an electrical potential (Figure 1.3), which can provide real-time, *in-situ*, monitoring of immunological reactions generating rapid, sensitive and reversible analytical signals.

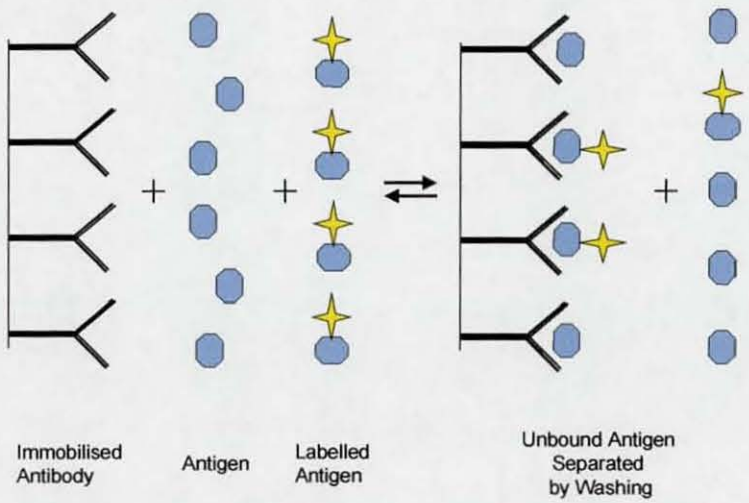
Figure 1.3 Schematic Diagram Showing the Operation of the Conducting Electroactive Polymer Based Immunosensor



Immunoassays, which use labelled reagents, can be categorised into those using labelled antigens or those using labelled antibodies. These can be further separated into antigen capture assays (Figure 1.4), antibody capture assays (Figure 1.5) or double antibody sandwich assays (Figure 1.6).

In an enzyme immunoassay (EIA), radioimmunoassay (RIA) and fluoroimmunoassay (FIA), the antigen is labelled (Figure 1.4). The labelled-antigen acts as a tracer to enable the total distribution of antigen to be determined.

Figure 1.4 Representation of Antigen Capture Assay

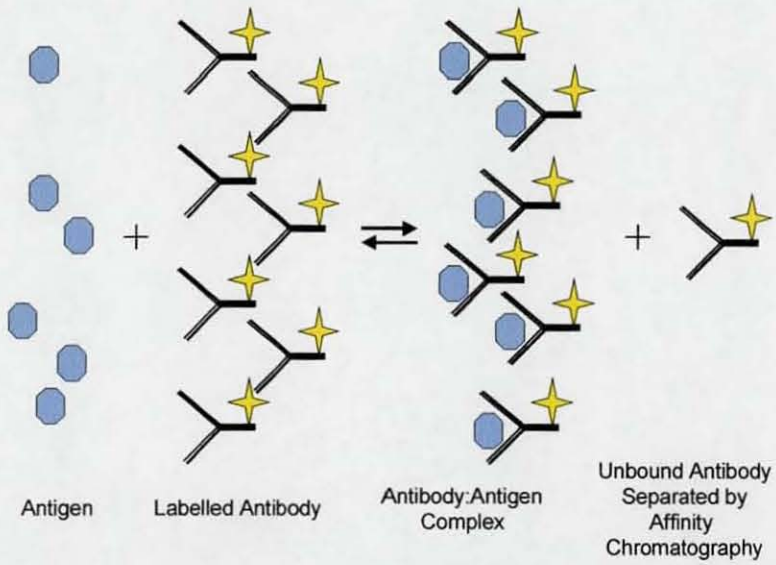


The antigen and tracer compete for the antibody, which has been immobilised on a solid surface. The amount of tracer and antibody are kept constant, and in limited concentration giving a competition assay. If the concentration of antibody is too great then all the antigen and tracer will be bound at equilibrium. Thus, any variations in the concentration of antigen in the sample are not detected. Classically, competitive immunoassays use tracers and antibodies in proportions that result in partial binding of the tracer in the absence of antigen. The measurement of the bound tracer results in a high signal, which decreases with increasing antigen concentration.[87] Therefore in EIA, RIA and FIA the signal detected from the enzyme substrate, radioisotope, or fluorophore is inversely related to the concentration of antigen in the sample.

RIA, EIA and FIA immunoassays are the most common assay formats used in clinical laboratories.[88] Clinical analysts use RIA because clinical samples rarely contain radioactivity that could interfere with the analysis. However, the inconvenience of handling and disposing of radioactive residues stimulated the development of immunoassays that are based on other labelling techniques, such as enzyme or fluorescence labels. Generally, EIA and FIA methods are sensitive and selective but they are often limited by the need for separation steps and the requirement of secondary labelling and introduction of secondary reactants.

Immunoassays such as the immunoradiometric assay (IRMA) or the immunofluorometric assay (IFMA) have labelled antibodies. They differ from RIA and FIA assays in that they only require two reactants instead of three (Figure 1.5).

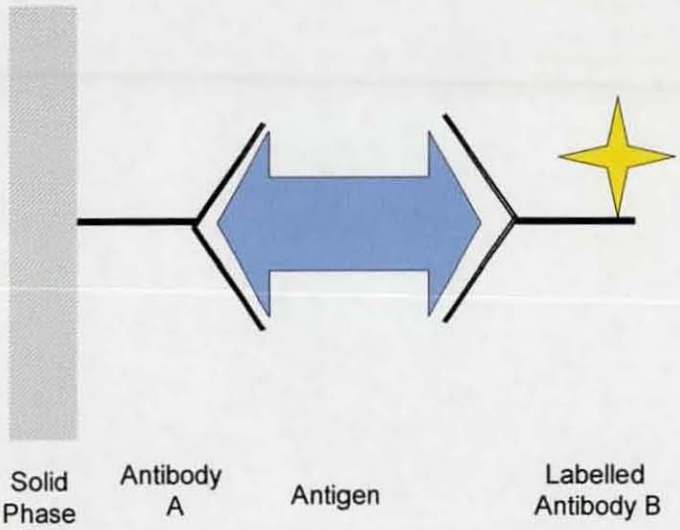
Figure 1.5 Representation of an Antibody Capture Assay



Excess-labelled antibodies are added to ensure the entire antigen is bound. The antigen bound antibodies are separated from the free fraction by affinity chromatography. The counts, or fluorescence intensity, are directly related to the total concentration of antigen present. IRMA and IFMA are defined as reagent excess, non-competition, assays.

The double-antibody sandwich assay was developed for large molecules with more than one antigenic determinant (Figure 1.6).[89]

Figure 1.6 Representation of a Double Antibody Sandwich Assay



The method usually employs two monoclonal antibodies, which have a high affinity for different epitopes on the antigen. The antigen is incubated with an excess of antibody, which is immobilised onto an insoluble solid support and is specific for one antigenic epitope. After careful washing, an excess of a second labelled antibody that is specific for a second determinant is added.

Kakabakos *et al.* described a sandwich assay that used antigen-specific antibodies immobilised onto polystyrene to detect lutropin, follitropin, choriogonadotropin and prolactin in human serum.[90] They used biotinylated antibodies as tracers. These reacted with europium labelled streptavidin and enabled fluorescence detection. Due to the antibody specificity the samples do not need prior purification.

Multiple antigen immunoassay systems have been commercially available since 1989, when Donohue *et al.* described a sandwich assay to detect antibodies to infectious agents in human serum.[91] In this assay antigens of the infectious agents are immobilised in distinct regions on a nitrocellulose membrane and anti-human antibody conjugated to horseradish peroxidase is used as the tracer.

The double-antibody method for the detection of antigens is the most common ELISA format used and has a detection limit of 0.1 – 1.0 fmol.[74]

1.5 Considerations for Immunoassay Design

1.5.1 Antibody Availability

The dominant reactant in all immunoassays is the antibody since this determines the specificity and ultimate sensitivity. The major requirement when developing an immunoassay is the ready availability of antibodies specific to the antigen of interest.

Specificity is defined in terms of the ability of an antibody to produce a discriminatory response only for the antigen of interest. The discriminatory response is a consequence of the inherent nature of the immune system, which can produce an almost limitless variety of binding sites on IgG. These binding sites have a very strong affinity for specific chemical structures or parts of structures on the antigen. However, since the same or similar antigenic determinants may exist within other molecules, there is the possibility of an antibody exhibiting cross-reactivity.

Cross-reactivity is dependent upon the quality of antibody or antiserum, which, in turn, is dependent on the reproducibility of the hapten-protein conjugate prepared for immunisation.[75] The specificity of binding free hapten to antibodies is determined largely by the structural features of the hapten and distance from the point of attachment to the protein immunogen.[92]

The problem of antigen cross-reactivity and the ability to minimise such cross-reactivity is particularly important in immunoassays. The use of affinity-purified antibodies from polyclonal antisera can reduce the problem of cross-reactivity, as can the use of monoclonal antibodies that have been tested for cross reactivity early in their production.[93]

A wide range of selective antibodies can now be produced for a variety of antigens.[84,75]

1.5.2 The Solid Phase

The immobilisation of antibodies or antigens on solid-phase materials has been used in many areas such as purification, diagnostic immunoassays and immunosensors.[94] In a standard ELISA assay the antibody is

immobilised onto the polymer surface of the microtitre well in which the enzymic reaction takes place. The type of plastic used is important since different antibodies will be adsorbed to a greater or lesser degree on different surfaces. The solid phase must be of uniform composition and structure to ensure equal antibody adsorption. The choice of microtitre plates includes polystyrene, polypropylene and polyvinylchloride; polystyrene being the most commonly used.

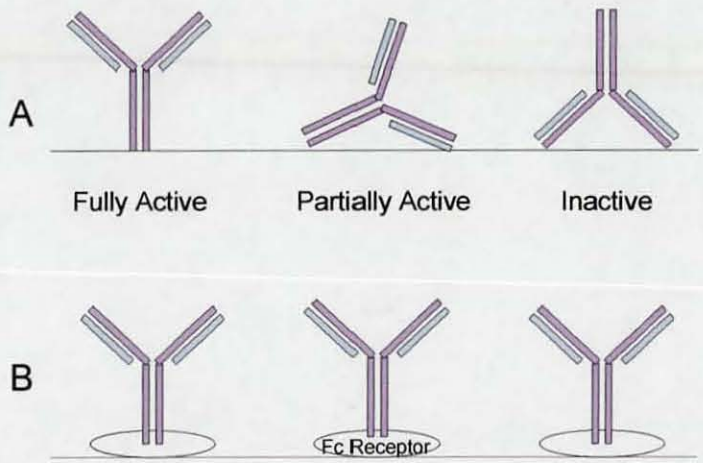
Adsorption of the antibody onto the solid phase occurs as a result of hydrophobic interactions between non-polar protein residues and the non-polar plastic matrix, or the antibodies can be coupled through their amino, carboxyl or carbohydrate residues.[95,96] This adsorption process does not involve any covalent interaction with the solid phase. The even coating of the wells will depend in the concentration of the antibody, the coating time, pH and temperature of incubation.[97]

Several other types of polymer matrices can be used as the solid phase for an immunoassay, they include nylon, polyvinylidene fluoride (PVDF), polymacron and cellulose such as nitrocellulose (NC), cellulose acetate and regenerated cellulose.[98,99] Adsorption of antibodies onto nitrocellulose and plastic gives non-covalent interactions and a random orientation onto the surface that could hinder antigen binding (Figure 1.7A).[94,100]

The mechanism for binding to nylon, however, is slightly different. The antibodies bind by electrostatic interactions between the amine groups of the antibody F_c region to the modified nylon surface (Figure 1.7 B).[101] This orientates the antibodies on the surface making the binding sites accessible to the antigen.

When antibodies are covalently attached to solid supports, their specific binding capacity is usually less than when in solution.[94] In addition to the orientation of surface-bound antibodies other factors can influence the antigen binding such as steric hindrance by adjacent antibodies. This creates over-crowding and leads to a decrease in antigen binding efficiency.[102]

Figure 1.7 Representation of Antibody Binding to Nitrocellulose (A) and Nylon (B)



In choosing a particular nitrocellulose membrane for an immunoassay, the role the membrane plays in the assay must be considered. Nitrocellulose membranes with pore sizes from 0.2 to 8 μm are commonly used. The larger pore size membranes have a lower surface area and therefore lower antibody-binding capacity. This yields a rapid filtration rate and so produces a fast assay. Conversely, a smaller-pore membrane can create a more sensitive assay.[98]

1.5.3 Non-Specific Binding

One problem frequently encountered when setting up an immunoassay is non-specific binding. Non-specific binding of the antigen may occur directly on the solid support surface where no antibody is present. Also, during the immobilisation procedure on the solid surface, non-specific binding of the antibody can occur. The latter case leads to heterogeneities on the immunoassay solid surface. There is a balance inherent in the practical use of immunoassay systems. Place *et al.* estimated that in an immunoassay, an acceptable response time of the order of a minute or less should be obtainable for μM antigen concentration levels.[103] However, the presence of non-specific binding will increase these times.[77]

As a general precaution against non-specific binding, wetting agents such as non-ionic detergents are normally added to all diluents and washing buffers used in the assay procedures. These do not interfere with antigen-antibody reactions but prevent new hydrophobic interactions between antigens and the solid phase. Non-specific binding may be minimised by the addition of a non cross-reacting protein at the time of antibody coating or as an extra coating step once the antibody coating is complete.

1.5.3 Antibody and Antigen Conjugates

A number of enzyme-linked antibodies are commercially available, in particular a number of enzyme-linked anti (species) IgG antibodies. These are particularly useful when developing a double antibody sandwich assay. However, situations do arise when one has to prepare appropriate conjugates. The major issue to be considered is the use of labels that do not disrupt the antibody-antigen binding mechanism.

A number of different enzymes have been used for antibody labelling but the two most commonly used are alkaline phosphatase and horseradish peroxidase. Both enzymes are readily available, cheap, have high specific activities, are stable with long shelf lives and can be readily conjugated to proteins while retaining their enzymic activity.

For antibodies the total immunoglobulin fraction of sera can usually be labelled rather than having to purify the antibody by immunosorbent methods. The conjugation of enzymes to antibodies may follow a one-step or two-step method. In the one-step method both components and a cross-linking agent, such as 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC), are mixed together. In the two-step procedure, one of the two components is reacted with the coupling agent then purified. This activated product is added to the second component. The optimal condition for conjugating two proteins must generally be determined by trial and error. Most enzyme conjugation methods give rise to a heterogeneous mixture of products that must be purified before use in the immunoassay.

Another major inhibiting factor affecting the immunoassay sensitivity is the relatively large molecular size of the enzyme label. Steric repulsions between the label and antibody can affect the selectivity of the antigen-antibody binding.[104] The use of labels with a smaller molecular size such as fluorophores, biotin, or adsorbed colloidal particles (gold or carbon), should improve detection sensitivity.[93,105] These labels have a molecular weight of less than 1000 Daltons. Being approximately 1/100th of the molecular size of antibodies, these labels will not sterically interfere with antigen-antibody binding. Patonay et al have suggested that an immunoassay that uses antibodies labelled with near-infrared laser dyes can have sensitivity comparable to that available with ELISA techniques.[105]

1.6 Fluorescence

Fluorescence is the emission of a longer wavelength radiation by a substance as a consequence of absorption of energy of a shorter wavelength. This occurs by a series of steps from a higher energy level returning to the ground level.[106,107]

1.6.1 Absorption and Emission of Radiation

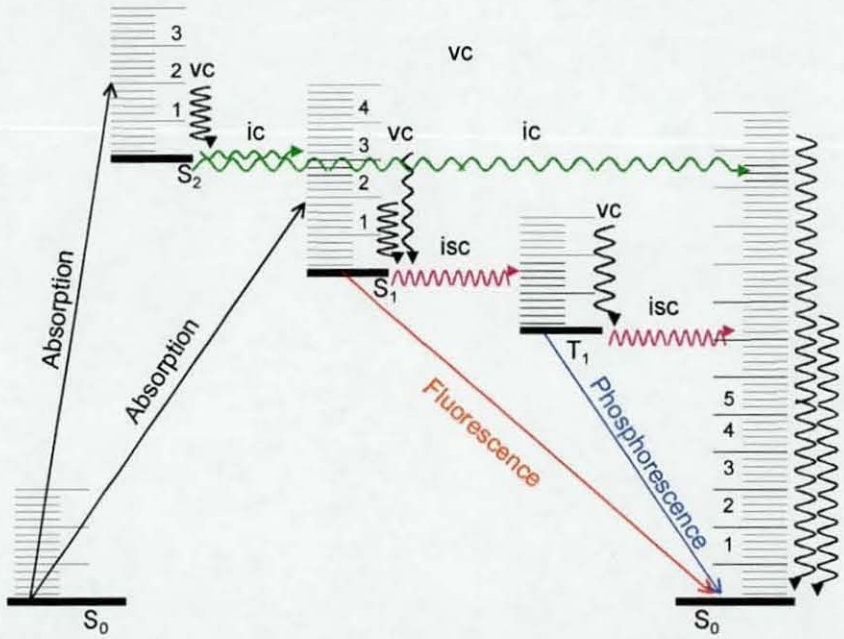
The absorption of radiation by an atom or molecule can lead to the production of an electronically excited species that can exhibit different chemistry than that of the ground state species. The method by which the excited species assimilates that energy, in rotational, vibrational or electronic modes, depends on the wavelength of the incident radiation.[108] Species can exist only in certain defined, discrete energy states and a transition between two such states has defined energy. As a result, the quantisation of energy levels for each individual species exhibits specific absorbed and emitted energies, and frequencies of radiation.

The distribution of electrons amongst the molecular orbitals gives the electronic configuration of a molecule. Each electronic configuration has orbital angular momentum and spin angular momentum, which determines the electronic state of the molecule.[109] Most molecules that exist naturally are stable and not very reactive because their electrons have paired spins and are closed shell molecules.[110]

Absorption of a photon by a molecule excites the electrons from the ground state, S_0 to a higher vibrational energy level in the upper singlet state, S_1, S_2, S_3, (Figure 1.8).[111,112] The electrons remain paired and may be in a vibrationally excited state where $V > 0$. In this excited state the molecule is subjected to collisions with its environment, its thermal energy is discarded by non-radiative means such as release of heat. This vibrational cascade succeeds in lowering the molecules' vibrational energy, relaxing to the lowest vibrational level of the first excited singlet state, S_1.[113] From this state most molecules will return to the ground level without emitting light, the excess energy being lost by internal conversions

or various quenching processes. Alternatively, as the electrons move from S_1 to S_0, a photon of defined energy can be emitted, fluorescence.

Figure 1.8 Jablonski Diagram Showing Absorption, and Emission Processes of Fluorescence and Phosphorescence[114]



S_0 (ground singlet state) refers to the electronic and vibrational transitions found when the electrons of a molecule are in σ and π bonding orbitals. S_1 (first singlet level) and T_1 (first triplet level) refer to electronic and vibrational transitions found in the σ^* and π^* antibonding orbitals. VC is vibrational cascade, IC internal conversions and ISC intersystem crossing.

Fluorescence is rapid having a first order decay with a lifetime of nanoseconds. It has a longer wavelength and lower energy than the absorbed photon. The difference between these excitation and emission wavelengths is the Stokes' shift.

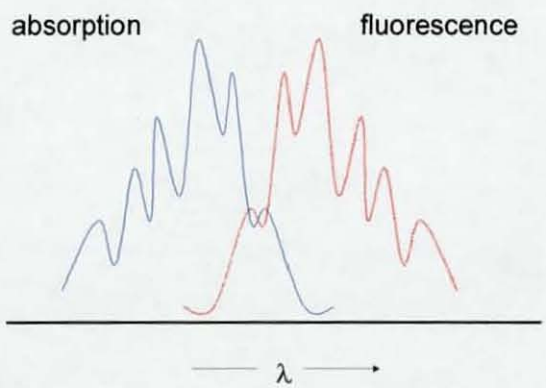
Electrons in the S_1 state may also undergo intersystem crossing to the first excited triplet electronic state, T_1. The subsequent radiative transition from T_1 to S_0 is phosphorescence, which has a lifetime of 10^{-3} - 10^{2}). The S to T transition is forbidden. However, phosphorescence is made possible as a result of spin-orbit coupling and the triplet level lying below S_1.[**110]

** Hund's Rule

1.6.2 Fluorescence Intensity

Fluorescence usually originates from the lowest excited singlet state $S_{1(v=0)}$, even though, through absorption the electrons may initially populate a higher singlet state. For many organic molecules the $S_{1(v=0)}$ to $S_{0(v=0)}$ transition is the most intense and the maximum intensity in both absorption and emission spectra correspond to the same transition[††]. The upper and lower electronic states of such organic molecules are a similar size and shape, the vibrational spacing will be the same and the absorption and fluorescence spectra will be mirror images (Figure 1.9).

Figure 1.9 Representation of Absorption and Emission Spectra



Fluorescence intensity (I_f) in dilute solutions is directly proportional to the intensity of the excitation light source and solution concentration and is given by Equation 1.2:

$$I_f = \Phi I_0 (1-10^{-\varepsilon bc}) \qquad \text{Equation 1.2}$$

Φ is the quantum efficiency, I_0 is the incident radiant power at the wavelength of interest, ε is the molar absorptivity, b is the cell path length and c is the molar concentration.[115]

When εbc is very small Equation 1.2 approximates to Equation 1.3:

$$I_f = 2.3\Phi I_0 \varepsilon bc \qquad \text{Equation 1.3}$$

[††] Franck-Condon Principle

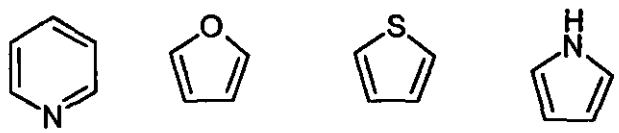
In order to emit radiation as fluorescence, a molecule must first absorb radiation. The higher the molar absorptivity (ε), the more intensely fluorescent a compound will be. Because fluorescent emission in organic compounds comes predominately from the $S_{1(v=0)}$, it is often found that the quantum efficiency (Φ) is independent of the excitation wavelength.[115] For 'highly fluorescent' molecules, such as fluorescein, under some conditions Φ approaches one.

In theory, the observed fluorescence intensity, which is only a fraction of I_f because of instrumental factors, is proportional to both the solute concentration and to the intensity of the exciting light source at the absorption wavelength. Suggesting the more intense the light source the greater the fluorescence. However, in practice a highly intensive radiation source can cause photodecomposition of the sample, therefore a moderate intensity is used.

1.6.3 Structure – Fluorescence Relationships

In most organic molecules of spectroscopic interest only the π-electrons and n-electrons are considered when determining the likelihood of fluorescence. The types of molecules that are most likely to fluoresce are those with delocalised π-orbital systems. Compounds containing isolated π-bonds exhibit π-electron transitions in the vacuum ultraviolet spectral region, and super-delocalised systems have π-electron spectra within the near ultraviolet to near infrared range.

The most intense and useful fluorescence is found in compounds containing aromatic functional groups with low energy π-π^* transition levels and most un-substituted aromatic hydrocarbons fluoresce in solution. However, the simple heterocycles such as pyridine (4), furan (5), thiophene (6) and pyrrole (7), do not show fluorescence.



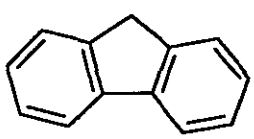
(4) **(5)** **(6)** **(7)**

This is due to the presence of the heterocyclic atom. In pyridine and pyrrole, the lowest energy electronic transition is thought to involve an n-π^* system, which rapidly converts to a triplet state and prevents fluorescence.

In a π-bond the distribution of electronic charge is concentrated above and below the plane of the σ-axis. These π-bonding electrons are free to move within the molecule and can be delocalised between several atoms. The number of rings and their degree of conjugation affects the quantum efficiency (Φ), which usually increases with increased π-electron conjugation.

1.6.4 The Effect of Structural Rigidity [76]

It is found that fluorescence is favoured in molecules that possess rigid structures, for example, when measured under similar conditions the quantum efficiency (Φ) for fluorene **(8)** and biphenyl **(9)** are 0.99 and 0.23 respectively. The difference in behaviour appears to be mainly as a result of the increased rigidity caused by the bridging methylene group in the fluorene. In addition, enhanced intensity results when fluorene is adsorbed on a solid surface. In these circumstances the added rigidity provided by the solid surface may account for the observed effect.



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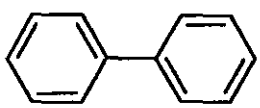


(9)

Lack of rigidity in a molecule causes an enhanced internal conversion rate and consequently an increase in the likelihood for radiationless decay.

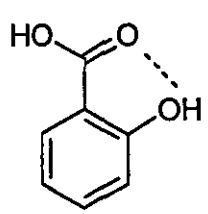
1.6.5 Effect of Substituted Groups

In a number of molecules the absorption of a photon can be traced to the excitation of the electrons of a small group of atoms. For example, when a carbonyl group is present in a molecule, absorption at about 290 nm is normally observed, although the precise absorption depends in the nature of the rest of the molecule. Groups of this kind are called chromophores, from the Greek for colour bringer, and their presence often accounts for the colour of materials.[113]

The type of substituted groups plays an important role in the nature and extent of fluorescence. Fluorescence yields of aromatic and heterocyclic hydrocarbons are usually altered by ring substitutions.[116,117,118] The fusion of benzene rings to heterocyclic nuclei result in an increase in the molar absorptivity (ε) and substitution on a benzene ring causes a shift in the absorbance wavelength (λ_{max}) and a corresponding change in fluorescence.[76] Groups that generally increase fluorescence are electron donating groups such as OH, OCH_3, NH_2, NCH_3 and $N(CH_3)_2$. It follows that those that diminish fluorescence are electron withdrawing. These include CHO, NH_3^+, $NHCOCH_3$, and COOH groups.[119]

Halogen substitution causes a decrease in fluorescence, a greater decrease is seen with increasing atomic number. This is thought to be due to the heavy atom effect, which increases the probability of intersystem crossing to the triplet state.

Intramolecular hydrogen bonds may effect the fluorescence, but this is often unpredictable. In salicylic acid **(10)**, for example, hydrogen bonding between carbonyl groups and the phenolic hydroxyl groups results in an enhanced fluorescence, whereas this type of bonding in proteins may quench the fluorescence.[120]



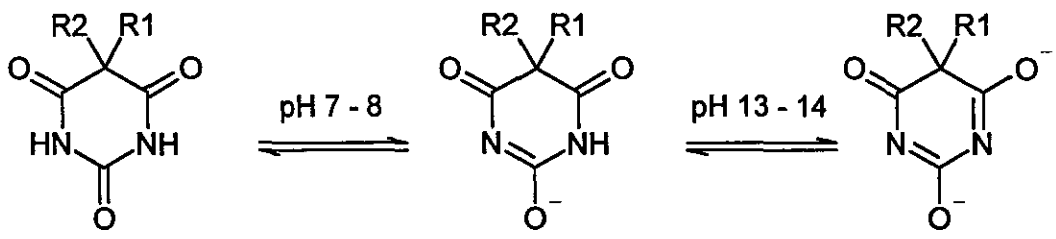
(10)

1.6.6 Temperature, Solvent and pH Effects[121,122,123]

The quantum efficiency (Φ) for most molecules decreases with increasing temperature. This is due to an increase in the frequency of molecular collisions at elevated temperatures, thereby increasing the probability for deactivation by external conversions.

A decrease in the solvent viscosity increases the probability of external conversions that lead to decreases in quantum efficiency. The polarity of the solvent exerts and important influence. The energy of n-π* transitions is often increased in polar solvents, while that for π-π* transitions decreases. These shifts may occasionally be large enough to lower the energy of the π-π* to below the n-π* and thereby enhance the fluorescence. Fluorescence is decreased in solvents containing heavy atoms, for example, carbon tetrachloride and ethyl iodide. The effect is similar to that which occurs when heavy atoms are substituted into fluorescent compounds, giving an increase in intersystem crossing

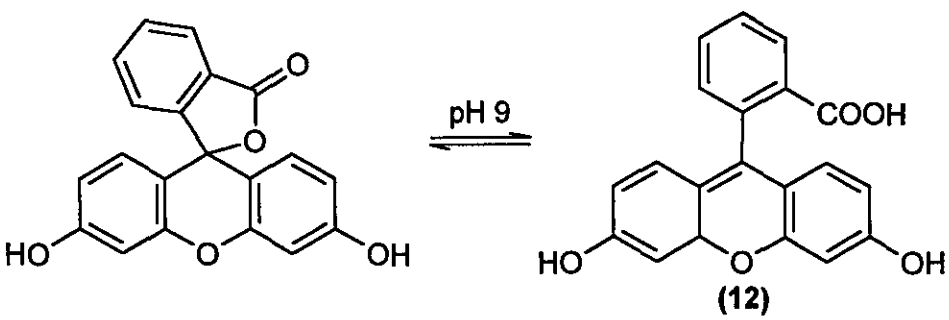
A large number of fluorescent compounds contain ionisable groups. In these compounds it is common to find that only one ionic form is fluorescent and that pH control is important, for example, in compounds such as oxybarbiturates **(11)** and fluorescein **(12)** where the fluorescence increases with increasing pH.



Unionised **(11)** Non-fluorescent

Mono-anion

Dianion Fluorescent



(12)

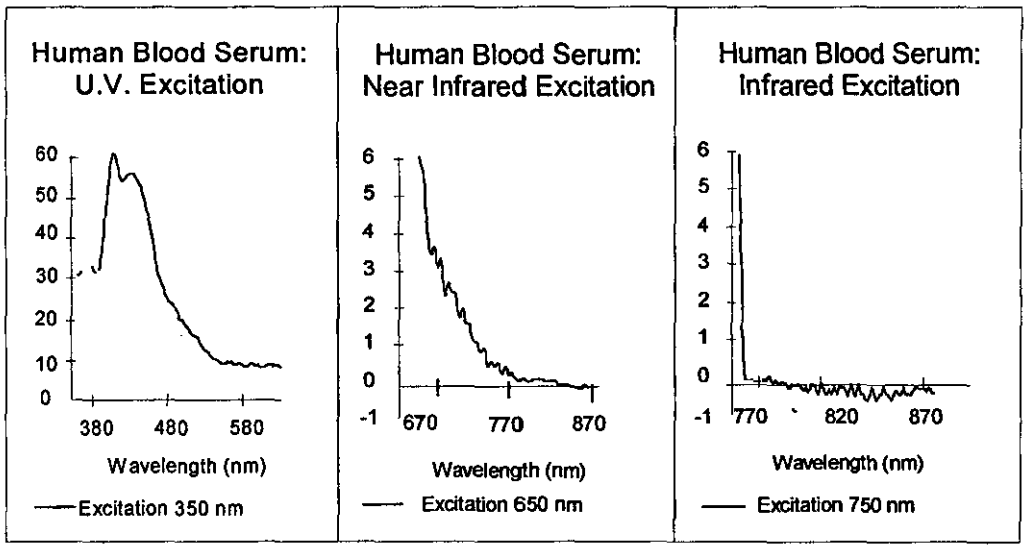
1.7 Fluorophores

1.7.1 Introduction

Fluorescence spectroscopy has been widely used in immunoassays since 1955.[70] The principle features of these assays are their spectral selectivity and sensitivity, due to the detection of a small emission signal against a zero or near zero background.

A significant number of dyes are commercially available for use as fluorescent labels, however, the absorption maxima for most of these are below 600 nm. UV/visible fluorophores such as fluorescein **(12)**, rhodamine and Texas Red have been the most commonly used. These were successfully attached to a variety of biomolecules such as enzymes, antibodies and nucleic acids for use in immunoassays, flow cytometry and protein sequencing.[124] Unfortunately, these labelling reagents are far from ideal as they suffer from reduced sensitivity because of high background fluorescence.[125] These background interferences originate from endogenous fluorescent species and from Raman and Rayleigh scattering, which are most significant in the UV/visible region of the spectrum (Figure 1.10).[126]

Figure 1. 10 Emission Spectra of Human Serum Albumin - Excited at Different Wavelengths



Fluorescence interference from an endogenous fluorophores, contained in a complex sample matrix, generate the most substantial background effect. Substances found in blood plasma for example, proteins and amino acids, have fluorescence emissions at 350 nm, and protein-bound bilirubin gives an emission peak at 520 nm.

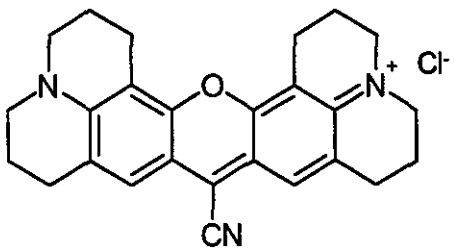
Most fluorescence experiments that involve biological samples are performed in aqueous conditions, which gives rise to the problem of Raman scattering. When an excitation wavelength of 350 nm is used a Raman signal appears at 397.5 nm, which can interfere with signals from other fluorophores. Rayleigh scattering is present in all solution based fluorescence experiments and is due to the solvent. Its effect can be minimised by the use of filters and monochromators and by the right-angled optical arrangement used in most fluorimeters.

Autofluorescence of glass, solvents or impurities can greatly reduce the potential sensitivity of the analysis. Yet fluorescence detection in the red and near infrared spectral region has proved to reduce this problem.[127] Near infrared fluorophores possess spectral properties which are observed between 700 and 1200 nm a region characterised by little interference from biomolecules. The fluorescence of impurities is diminished, since the number of compounds absorbing light and fluorescing is very small, and Raman scattering is reduced because the intensity is proportional to λ^{-4}.[128] Thus the major interference in the near infrared region is due to noise generated from the detector. The sensitivities of near infrared assays are usually detector limited as opposed to background limited. This situation has improved significantly with the development of semiconductor-based detectors, photodiodes, and recently available near infrared laser diodes.

The spectral range makes these fluorophores suitable for excitation with inexpensive laser diodes, which enhances the observed fluorescence signal.[124] Consequently, near infrared fluorescent labels are being increasingly used in bioanalytical applications.[129,130,131,132,133]

1.7.2 Near Infrared Fluorophores

Molecules with near infrared spectral properties can be natural dyes such as porphyrins (λ_{max} = 619 nm) or synthetic dyes such as xanthene **(13)**, oxazine **(14)**, phthalocyanine **(15)** and polymethine cyanine **(16)** dyes.[134] These groups of organic compounds give intense fluorescence in the near infrared region.



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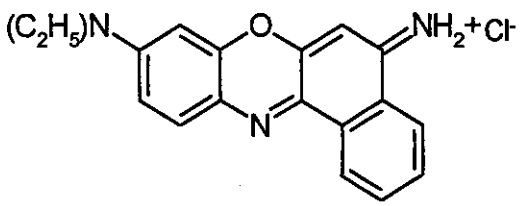

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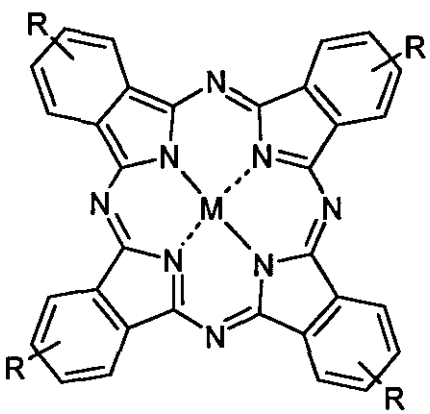

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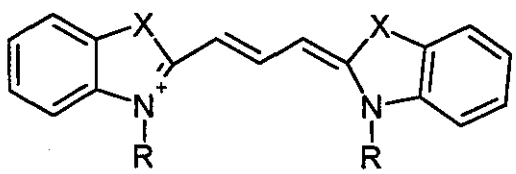


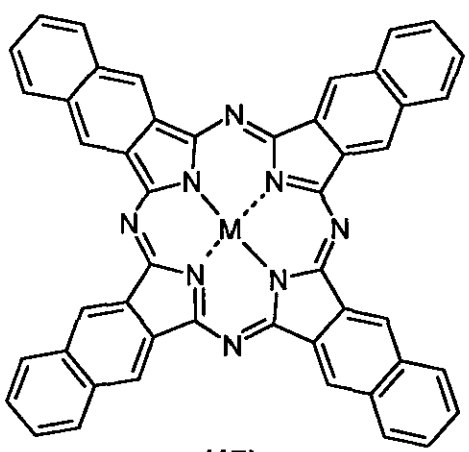
(16)

Oxazines such as Nile blue **(14)** have been used as labels for proteins. Their absorption maxima shift to longer wavelengths with an increasing number of alkyl or aryl substituents on the two-amine functionalities. Unfortunately, a decrease in the quantum efficiency is seen in aqueous solutions, an effect that is also seen on reaction of the primary amine group. Consequently, commercially available oxazines are less useful as fluorescent labels.[135]

Phthalocyanine dyes **(15)** have a structure analogous to the natural porphyrins and have become important colorants as dyes and pigments. In the search for infrared absorbing dyes for optical recording media,

phthalocyanines (λ_{max} = 690 nm) were the first candidates to be considered and have attracted attention as functional dyes in various fields, such as laser disc memory and photodiodes.[136] Metal-free phthalocyanines absorb at 698 nm in a 1-chloronaphthalene solution and at 772 nm in the solid state. The metal complexes generally absorb at much shorter wavelengths than the metal-free dyes, but some, such as lead phthalocyanine, absorb at much longer wavelengths. Metalation reduces the electron density at the inner nitrogen atoms, and as the charge transfer in the compound occurs from the centre towards the outside, a hypsochromic shift in wavelength is observed. The extent of the λ_{max} shift to shorter wavelength depends on the electronegativity of the metal.

Phthalocyanine dyes serve as precursors for naphthalocyanine isomers with absorbance maxima ranging from 720 to 800 nm. These are produced by annelation of a phenyl ring and give a bathochromic shift in wavelength. The naphthalocyanine dyes are planar, rigid molecules, which undergo an excitation process resulting in the population of the first singlet state S_1. The absorption spectra of 2,3-naphthalocyanines **(17)**, are consistent with $\pi - \pi^*$ transitions in the energy region above 769 nm.[137]



(17)

They are very stable, resist degradation by light, heat and oxidation, but have limited solubility in protic solvents and relatively small Stokes' shifts. However, the series of 2,3-naphthalocyanine derivatives is potentially very important with electro-optical applications in areas such as optical

recording media, organic photoconductors, colour filter dyes and photosensors. They have been modified to improve solubility through the introduction of branched long-chain alkyl groups into the naphthalene rings and trialkylsiloxysilane into the central core.

Polymethine cyanine dyes are smaller molecules, usually less photostable than naphthalocyanine dyes, but generally have higher aqueous solubility. They are particularly interesting because they can be tailored for different applications due to the ability to predict their spectral properties. They can be tuned to the desired wavelengths by altering the heterocyclic nucleus and the number of double bonds in the methine chain. By changing the functional groups, several multi-colour derivatives of these dyes have been made, and used for different tasks including pH determination, metal ion analysis, DNA labelling and used in optical recording media and in laser sensitive lithograph printing plates.[138,139]

To date, the phthalocyanine **(15)**, polymethine cyanine dyes **(16)** and naphthaolcyanines **(17)** have been the most widely applied near infrared dyes.[140] They possess large molar absorptivities (ε), usually between 100,000 and 200,000 $Lmol^{-1}cm^{-1}$, acceptable quantum efficiencies and are strongly fluorescent.

1.7.3 Polymethine Cyanine Dyes

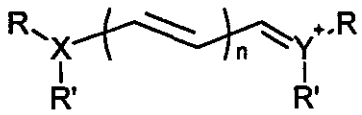
There has been significant interest in preparing dyes that show electronic absorptions further into the infrared region. Cyanine dyes have proved to be exceptionally useful in this respect.[141]

Almost all of the commercially available cyanine chromophores were developed by using the concepts of the resonance theory.[142,143,144] The colour and constitution of cyanine dyes may be understood through consideration of their component parts these being the chromophoric system, the polymethine chain and the terminal groups. The way in which these groups interact can give rise to differing physical and spectral properties. Employing resonance theories to the π-electron systems of cyanine dyes enables the properties such as the colour, absorption

maxima (λ_{max}), the molar extinction coefficient (ε), melting point and other colour-related properties of the dyes to be predicted.

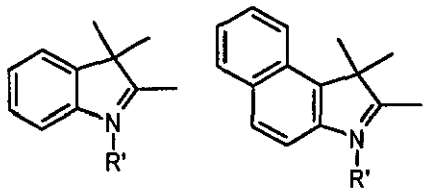
1.7.4 Effect of Structural Modifications

In general, polymethine cyanine dyes have two heterocyclic rings joined by a conjugated chain of carbon atoms, having the general structure **(18)**, and they absorb in a wide range of wavelengths from 340 to 1400 nm.



(18)

In the structure R denotes a heteroaromatic residue, such as indolenine or benzindolenine, n is a positive integer giving the compounds an odd number of ethylene residues, X and Y are usually nitrogen atoms and R' is a substituted alkyl chain.

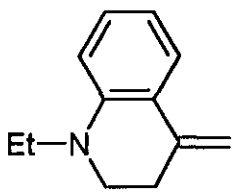


Indolenine Benzindolenine

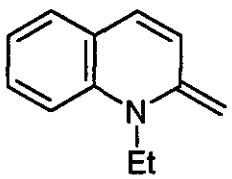
The absorbance maxima of cyanine dyes increase as the polymethine chain increases, when n>3 a near infrared absorption is exhibited, and for each ethylene group added, an increase of approximately 110 nm is observed.[127] The number of double bonds in the polymethine chain between the heterocyclic nuclei allows control of the colour of the fluorochrome.[104]

There are numerous non-symmetric cyanine dyes that absorb in the near infrared region. These contain two different heterocyclic end groups, one heterocyclic and one carbocyclic end group, or one heterocyclic and one noncyclic end group. In general, their absorption (λ_{max}) is displaced to shorter wavelengths compared to the average values of λ_{max} in the corresponding symmetrical dyes.

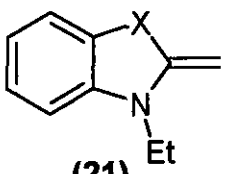
Symmetrical cyanine dye fluorophores of the type R-(CH=CH)-$_n$CH=R$^+$ are ionic in nature and the formal charges are located at the ends of the chromophore. Two extreme resonance structures are possible which are equivalent in energy. In general, the absorption consists of one strong maximum at long wavelengths followed by a smaller vibronic side band shoulder.[145] The λ_{max} of cyanine dyes is strongly affected by the nature of the aromatic group R, the electronic characteristics of the cyanine residue, the number of the ethylene units in the conjugating bridge and by steric hindrance.[146,147] The effect of the heteroatomic ring depends on the structure of R, for example **(19)**, **(20)** and **(21)**.



(19) λ_{max}=920 nm



(20) λ_{max}=818 nm



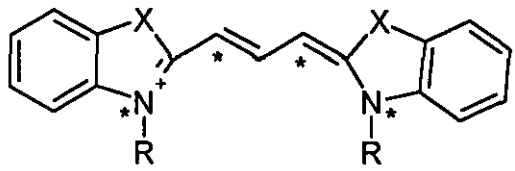
(21)

X =Se : 790 nm
S : 765 nm
CMe_2 : 741 nm
O : 695 nm

The absorption spectrum changes substantially when oxygen is substituted by sulfur in the heterocyclic ring **(21)**. The presence of sulfur broadens the absorption band and causes a bathochromic shift in λ_{max} and in addition increases the photostability. Increasing the basicity of the heteroatom and enlarging the π-conjugated system, causes a bathochromic shift.

The addition of electron-withdrawing or electron-donating groups to the fluorophore can produce one of two effects, a bathochromic or hypsochromic shift. Yasui *et al.* studied the effects of substituent on the fluorescence of trimethine dyes (n=1) and optimised the substitution parameters using the molecular orbital theory. Mapping the changes accompanying the first excitation revealed a decrease in the π-electron

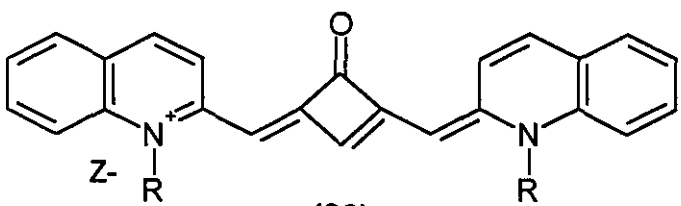
density (indicated as the starred positions in **(22)**) and a subsequent increase of electron density at the unstarred positions.



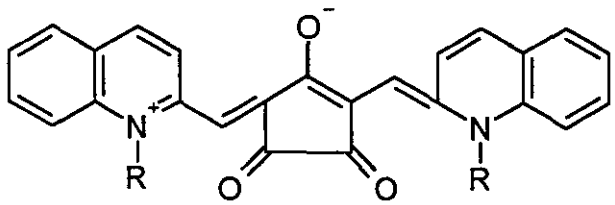
(22)

Introducing an electron-donating group at a starred position produces a bathochromic shift in trimethine (n=1), pentamethine (n=2) and heptamethine (n=3) cyanine dyes.

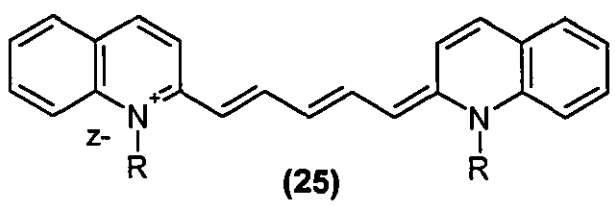
In pentamethine cyanine dyes, substitution at the *meso*-position of the conjugated methine chain can exert a bathochromic effect on the λ_{max}. An increase in phosphorescence caused by intersystem crossing is expected to occur when heavy atoms or a bulky group are introduced in the *meso*-position of the polymethine chain.[148] In compounds such as squarylium **(23)** and croconium **(24)** substitution at the meso position causes the absorption to shift into the infrared region relative to the pentamethine dye **(25).** The absorption spectra of infrared absorbing dyes can be shifted quantitatively in this manner.[149]



(23)



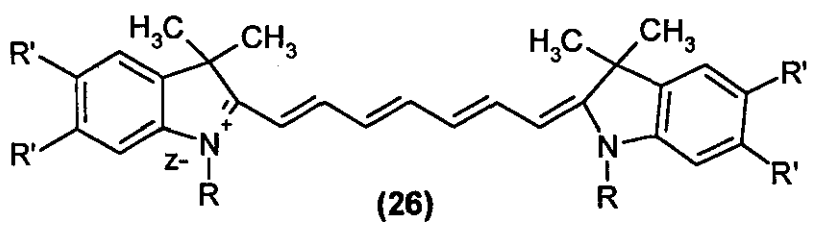
(24)



(25)

The lifetimes of fluorophores are correlated with the molecular structure. It is well known that rigid molecules dissipate less energy via internal conversions and tend to have longer S_1 lifetimes than molecules with freely rotating parts. Fortunately, dyes that have a short lifetime and higher photostability do not deteriorate upon a single excitation and have a reduced chance of photodecomposition.[145]

In heptamethine cyanine dyes the solubility in organic solvents is strongly affected by the nature of the N-alkyl substituent, the heteroaromatic ring and the counter anion. The N-alkyl substituent not only affects the solubility but also the aggregation of dye molecules that can cause strong light scattering.[150] The reactivity, charges, and solubility properties of basic heptamethine indolenine dye **(26)** can be readily controlled by placing appropriate substituents at the R position, and at R' on the benzene rings of the nucleus.

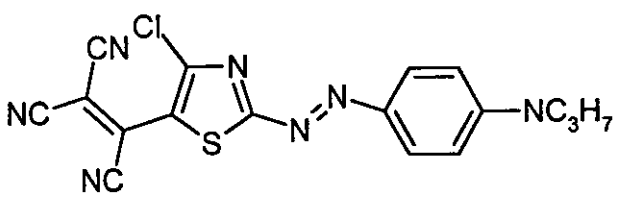


(26)

Suitable R substituents are carboxyl alkyl groups that can be activated to form succinimidyl esters; the spectra of the carboxylic acid containing dyes, and their activated esters, have narrow absorption and emission bands and a low vibronic shoulder on the short-wavelength side of the absorption peaks.[104,145]

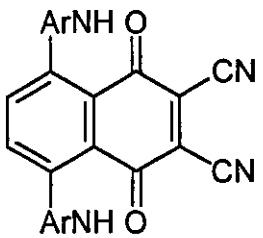
The addition of sulfonate groups at the R or R' positions will increase water solubility, with the introduction of the two or four sulfonate groups onto the ring, these dyes dissolve easily in water. The direct attachment of the sulfonic acid group to the chromophore aromatic system does not appreciably change the indolenine cyanine dye absorption and emission wavelengths and no evidence of new absorption bands, indicative of dimers and other dye-dye interactions are seen. Presumably, this is because the sulfonic acid groups on the ring structure make the fluorophores have less affinity for one another.

In general, the indolenine infrared absorbing dye **(26)** have poor lightfastness when compared with other dye chromophores such as azo **(27)** and quinone dyes **(28)**.



λ > 725 nm

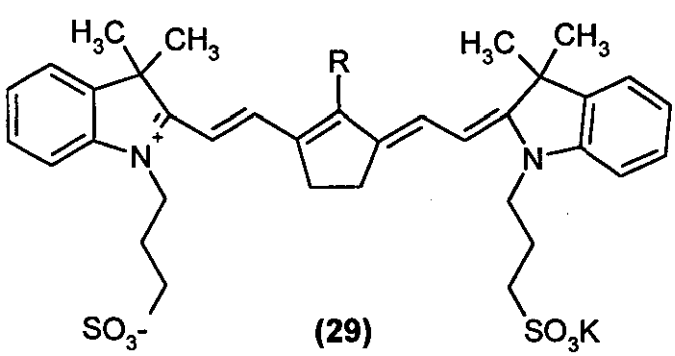
(27)



λ = 798 nm

(28)

The lightfastness of pentamethine and heptamethine cyanine dyes can be improved by incorporating a five or six carbon atom rings into the methine conjugated bridge **(29)**.



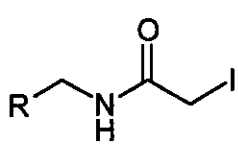
(29)

Rigid chromophores, in general, have a high efficiency of fluorescence. Fluorophore **(29)** was designed by Patonay and co-workers to contain a propane-1,3-diyl bridge for decreased conformational movement.[151] Due to the presence of sulfonate groups the dyes show excellent water solubility, and give solutions that are relatively stable even when exposed to light.[125]

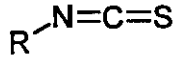
1.7.5 Fluorescent Labelling

Until 1993 the majority of fluorescent labelling was the non-covalent labelling of proteins through electrostatic adsorption using hydrophobic, non-functionalised polymethine dyes.. Successful labelling and good assay detection sensitivities were accomplished with these non-covalent labelled proteins, but problems occurred because of damage to size exclusion columns used to purify these labelled reagents.[133] To overcome this damaging problem covalent labelling with functionalised near infrared cyanine dyes was developed. The functional group could be attached directly to the dye chromophore (at R' position in structure **(26)**) or to the alkyl chains (at the R position), giving mono-functionalised or bi-functionalised labelling reagents.

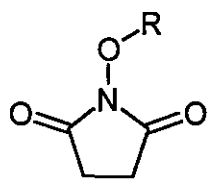
Waggoner *et al.* developed a new class of biological labelling reagents based on symmetrical cyanine dyes with iodoacetamide **(30)**, isothiocyanate **(31)**, N-succinimidyl **(32)** and hydrazide **(33)** groups for thiol, primary and secondary amines and aldehyde labelling, respectively.[132]



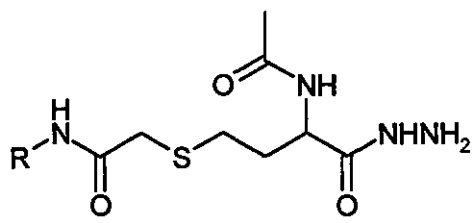
(30)



(31)



(32)

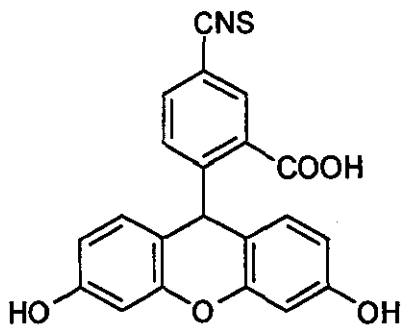


(33)

Gooijer *et al* successfully used dyes containing the iodoacetamide group **(30)** for the determination of the non-steroidal anti-inflammatory drug naproxen in saliva.[152] This was accomplished in two ways either coupling to the cyanine dye by direct linkage or by use of a spacer. They discovered that direct linking of the naproxen to the aromatic part of the

dye caused an extended conjugation of the π-system for the fluorophore. This resulted in a bathochromic shift of $\lambda_{max.}$, an increase in the Stokes' shift, and a significant reduction in the fluorescence quantum efficiency. Coupling via an alkyl substituent induced only small wavelength shift and the quantum efficiency was relatively unaffected.

The highly reactive isothiocyanate group **(31)** can react with various types of compounds, particularly those with active hydrogen. The reaction takes place above pH 9.0, is spontaneous, and the isothiocyanate group reacts selectively with amines or alcohols to form stable thioureas or thiourethanes. Bhattarayya[130] used this principle to directly label nucleic acids with fluorescein isothiocyanate **(34)**, and Narayanan has successfully used this group to label DNA with fluorophore **(29)** which is used in DNA sequencing.[155]



(34)

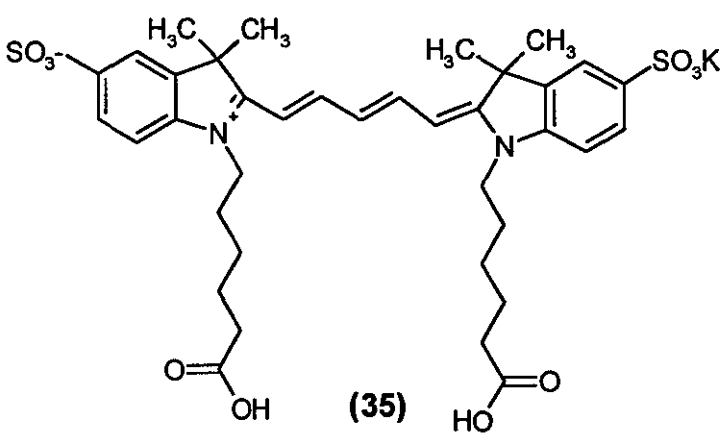
The succinimidyl ester group **(32)** is well known for its acylating properties and can be used for labelling primary and secondary amines at slightly alkaline pH.[153] An inherent disadvantage of this functional group is that in aqueous conditions succinimidyl esters undergo hydrolysis, which makes it impossible to derivatise low concentrations of antigen *in-situ*.

Gooijer has extensively used dyes that contain hydrazides to label aldehydes in their pre-column HPLC procedures.[128] They proposed that when labelling small antigens mono-functionalised dyes, containing a single reactive group, should to be used in preference to bifunctional dyes as complications from cross-linking can occur. The experience of Waggoner *et al.* has shown that cross-linking of proteins does not occur to a significant extent when using bi-functionalised dyes.[138]

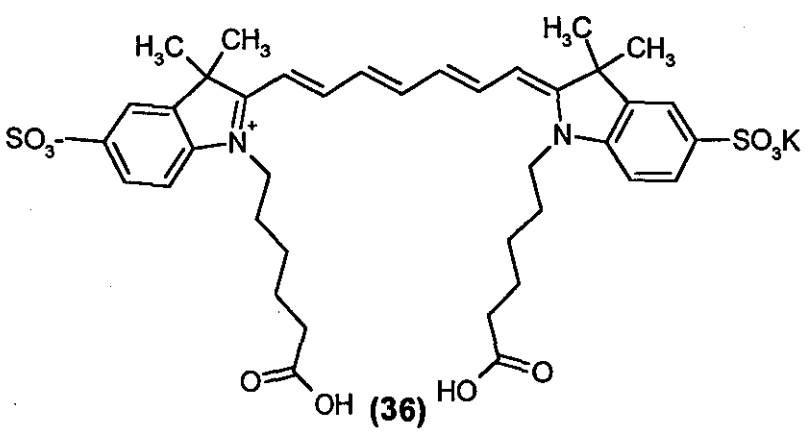
1.7.6 Ideal Fluorophores

The ideal fluorescent probe should allow maximum antigen detectability with the minimum disruption to the function of the labelled molecule. They will be water-soluble, maintain their fluorescent intensity at a physiological pH, should experience very little interference from endogenous fluorescent species, and have an excitation wavelength that is compatible with the output of commercial laser diodes. These requirements are fulfilled by several classes of polymethine cyanine dyes that fluoresce in the near infrared region.

Waggoner *et al* developed the pentamethine and heptamethine cyanine dyes Cy5.18.OH **(35)** and Cy7.18.OH **(36)** that have excitation wavelengths at 650 nm and 750 nm, respectively.



(35)



(36)

These sulphoindocyanine dyes contain negatively charged sulfonate groups on the indolenine base, which significantly reduces dye-dye interactions, and makes them highly water-soluble; they contain one or

two succinimidyl ester groups on the aromatic chromophore that enables the dye to be conjugated to amine containing materials; and the photostability of Cy5.18.OH **(35)** is comparable to that of fluorescein isothiocyanate **(34)**.[104]

The near infrared fluorophore derivatives of Cy7.18.OH **(36)** are initially more attractive than Cy5.18.OH **(35)** analogues because of their longer excitation and emission wavelengths. Unfortunately, they have not found widespread application because they are less chemically, thermally and photo-stable than the Cy5.18.OH **(35)** derivatives.

Patonay *et al.* synthesised an activated derivative of **(29)**. It contains sulfonate groups on the indolenine base for increased water solubility and has a single isothiocyanate group in the centre of the ring stabilised methine chain for amine or alcohol linking.[154] This dye has excellent solubility in water, the solutions being stable even when exposed to light; exhibits short fluorescence lifetimes, which suggest that they are relatively less susceptible to photo-destruction and photobleaching; has been reported to efficiently label proteins and produce stable protein adducts; has a high molar absorptivity coefficient, a relatively high quantum efficiency and produce bright signals. [154,155]

The absorbance and fluorescence properties of all the dyes alter on conjugation. When covalently bound to DNA, the absorbance and fluorescence maxima, quantum efficiencies, and Stokes shift of the fluorophore alter when compared with the native dye, indicating that some quenching of the dye by the oligomer occurs. However, efficient fluorescence and good stability has been observed for adducts of **(29)**, **(35)** and **(36)** with proteins.[155,156]

CHAPTER TWO

EXPERIMENTAL METHODS

2.1 Introduction

The experimental methods section is split into four main sections covering reagents, purification techniques, routine analyses, and instrumentation used throughout the research.

UV-visible spectroscopy was used to determine the molar absorption coefficients (ε), excitation wavelengths (λ_{max}), and concentrations of the various fluorophore conjugates. Traditional immunoassay techniques using sheep anti-sulfamethazine antisera have been used to study the antibody binding of the sulfamethazine-fluorophore conjugates, and fluorescence spectroscopy was used to give more information about the change in fluorescent activity of the fluorophore when conjugated to sulfamethazine.

2.2 Chemical Preparations and Storage of Solutions

Roy Jackman of the Central Veterinary Laboratory, Weybridge, provided the sheep anti-sulfamethazine antisera, which was purified by ammonium sulfate precipitation and stored in the freezer at -18°C.[157,158]

All chemicals were obtained from Sigma-Aldrich-Fluka Ltd (Poole, UK) and the solvents were from B.D.H. (Poole, UK), unless otherwise stated (Appendix 2). These were all used as supplied and stored as recommended.

The disposable plastic wear from was obtained from Sterilin Ltd (Stone, UK), Slide-a-lyser dialysis tubing came from Pierce and Warriner Ltd (Cheshire, UK), Autostat AHU10 Heat stabilised polyester was supplied by Autotype International Ltd. (Wantage, Oxon), and the double-sided Scotch™ tape came from 3M Company Ltd (UK).

The nitrocellulose, nylon, PVDF and polymacron membranes were supplied by Millipore Corporation (Bedford, MA, USA), Pall Gelman Ltd (Portsmouth, England), Whatman International Ltd (Maidstone, UK.), Schleicher and Schuell GmbH (Dassel, Germany) and Kalyx Corporation

(Ontario, Canada) (Appendix 4, Table A.4.1). These were used as supplied and stored as recommended.

2.3 Preparation of Buffers and Other Reagents

All buffers were made up in A-grade volumetric flasks using polished water with a conductivity 18.2 mΩ and stored in glass containers for up to 6 weeks, unless otherwise stated,

The pH measurements were performed using a Philips PW9420 pH meter, which was calibrated with the appropriate standards before use.

Gilson pipettes were used throughout and calibrated prior to use.

2.3.1 Phosphate Buffered Saline (0.1 M, 0.9%$_{w/v}$, pH 7.4)

Sodium dihydrogen phosphate (4.20 g), disodium hydrogen phosphate (10.41 g), sodium chloride (9 g) and water (750 ml) are added to a 1 L volumetric flask and the solutes dissolved. The solution is made up to the mark with water, and the pH adjusted by adding sodium hydroxide or hydrochloric acid.

2.3.2 Phosphate Azide Saline Buffer (0.1 M, 0.05%$_{w/v}$,pH 8.0)

Sodium dihydrogen phosphate (4.20 g), disodium hydrogen phosphate (10.406 g), sodium chloride (9 g), sodium azide (0.5 g) and water (750 ml) are added to a 1 L volumetric flask. The solutes are dissolved and the solution made up to the 1 L mark with water.

2.3.3 Sodium Carbonate Buffer (0.1 M, pH 9.3)

Solutions of sodium hydrogen carbonate (0.84%$_{w/v}$) and sodium carbonate (0.716%$_{w/v}$) are made in water. Sodium hydrogen carbonate solution (85 ml) and sodium carbonate solution (10 ml) are mixed together and the pH adjusted to 9.3 by the dropwise addition of sodium carbonate solution.

2.3.4 Sodium Borate Buffer (0.25 M, pH 8.5)

Disodiumtetraborate (9.525 g) is made up to 1 litre with water. 100 ml of this solution is added to hydrochloric acid (0.1 M, 30.4 ml) and made up to 200 ml with water. The pH is adjusted by dropwise addition of 0.1 M hydrochloric acid.

2.3.5 Sulfamethazine Standard Solutions

Sulfamethazine (0.0100 g) is made up to 100 ml with phosphate buffered saline (0.1 M, 0.9%, pH 7.4) with stirring and ultra-sonication to aid dissolving. The resulting solution has a concentration of 100 μg/ml, ($3.59e^{-4}$ M).

10 ml of 100 μg/ml sulfamethazine solution is diluted to 100 ml with phosphate buffered saline giving a 10 μg/ml solution, ($3.59e^{-5}$ M). 1 ml of 100 μg/ml sulfamethazine solution is diluted to 100 ml with phosphate buffered saline giving a 1 μg/ml solution, ($3.59e^{-6}$ M), equivalent to 1 ppm. The 1 μg/ml (1 ppm) solution is serially diluted to give standard solutions of the following concentrations:

Table 2.1 Dilution Factors and Concentrations of Sulfamethazine

Dilution factor (sulfamethazine/PBS)	w/v	Molarity	Ppb
½	500 ng/ml	$1.795e^{-6}$ M	500
¼	250 ng/ml	$8.975e^{-7}$ M	250
$^1/_{10}$	100 ng/ml	$3.590e^{-7}$ M	100
$^3/_{40}$	75 ng/ml	$2.692e^{-7}$ M	75
$^1/_{20}$	50 ng/ml	$1.795e^{-7}$ M	50
$^1/_{100}$	10 ng/ml	$3.590e^{-8}$ M	10

2.3.6 Carmazine Red Solution (0.05%$_{w/v}$)

Carmazine red (0.5 g) is made up to 100 ml with phosphate buffered saline (0.1 M, 0.9%$_{w/v}$, pH 7.4).

2.3.7 Tween-20 Wash Solution (0.05%$_{v/v}$)

Tween-20 (0.5 ml) is made up to 1 litre with tap water.

2.3.8 3,3',5,5'-tetramethylbenzadine Chromogenic Substrate[‡‡]

3,3',5,5'-tetramethylbenzadine A reagent solution (4.2 ml) and 3,3',5,5'-tetramethylbenzadine B reagent solution (14 ml) are mixed together as indicated by the manufacturer. This solution is equilibrated to room temperature and prepared immediately prior.

2.3.9 Saturated Ammonium Sulfate Solution

Ammonium sulfate (550 g) is added to water (600 ml) in a beaker and the solution is slowly heated until all the salt is dissolved, then the hot solution is filtered to remove any insoluble impurities. The solution is allowed to cool to room temperature and made up to 950 ml with water. Concentrated ammonium hydroxide is added to give pH 6.5 and the volume made up to 1 L with water.

2.3.10 Amido Black Staining Solution

Amido Black (Napthol Blue Black, 0.5 g), methanol (50 ml) and acetic acid (5 ml) are made up to 100 ml in water.

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‡‡ Reagents provided by Vetoquinol Ltd.

2.3.11 De-stain Solution

Methanol (50 ml) and acetic acid (5 ml) are made up to 100 ml in water.

2.3.12 Casein Blocking Solution

A stock solution is prepared by dissolving casein (1.0 g) in phosphate buffered saline (100 ml) to give a $10\%_{w/v}$ solution. This solution is autoclaved at 121°C for 20 minutes and stored at 4°C for up to 3 months. Immediately before use, the casein solution is diluted with phosphate buffered saline to give the required final concentration, as stated in the relevant experiment.

2.4 Purification Methods

2.4.1 Thin Layer Chromatography

Preparative thin layer chromatography purification was performed using Reverse $Phase_{C\text{-}18}$ TLC plates (60 Å, 250 μm, 20 cm x 20 cm) or K6 silica TLC plates (60 Å, 250 μm, 20 cm x 20 cm) supplied by Whatman International Ltd (Maidstone, UK). The solvent system used for each compound is specified in the corresponding experiment.

2.4.2 Gel Filtration Chromatography

Purification of compounds by gel filtration chromatography is performed using Sephadex G-10, G-25, G-50 heterogeneous gel filtration media for aqueous separations, using a media whose pore size is comparable to the molecules being separated, and Sephadex LH-20 heterogeneous gel filtration media for organic solvent separations. All gel filtration media, XK 26/40 columns, the peristaltic pump, UV detector, chart recorder and auto-sampler are supplied by Pharmacia Ltd (Milton Keynes, UK).

The Sephadex material is supplied as an anhydrous powder. The media is mixed with the relevant eluent solution and allowed to swell for 4 hours. The swollen gel is allowed to settle and excess solution removed, forming a slurry. The columns are mounted vertically and the dead space in the bed support and tubing are filled with elution solution, being cautious to remove all air bubbles. The gel slurry is poured carefully into the column and solvent is added until the column is full. An eluent reservoir is connected to the column and the flow started. For LH-20 gel using a chloroform containing eluent, the column is packed with the slurry, an adapter is inserted to retain this, and the column inverted before connection to the reservoir.

The newly packed column is eluted at a fast flow rate for several hours prior to use to allow uniform packing and when the column is smoothly packed it is ready for use.

After use, the columns are eluted for 45 minutes with eluent to clean the gel. SG columns are further eluted with phosphate azide saline buffer (PAS, 0.1 M, 0.05%$_{w/v}$, pH 8.0) in to prevent microbial growth. The connection tubing is sealed, ensuring that the reservoir of eluent above the gel stops the column from drying out, and the column stored at room temperature. The column is washed with elution buffer before being reused.

2.4.3 Purification of IgG[158]

Saturated ammonium sulfate solution (50 ml) is added dropwise to the antiserum (100 ml) stirring constantly. The serum solution is allowed to stand for 15 minutes and then centrifuged. The pellet is washed twice with saturated ammonium sulfate solution (100 ml, 40%$_{v/v}$) by centrifuging and discarding the supernatant each time. The pellet is dissolved in sodium chloride solution (100 ml, 0.9%$_{w/v}$) and re-precipitated with saturated ammonium sulfate solution. After washing twice with ammonium sulfate solution (100 ml, 40%$_{v/v}$) the pellet is re-dissolved in sodium chloride (100 ml, 0.9%$_{w/v}$) and re-precipitated for a third time with saturated ammonium sulfate solution (33%$_{v/v}$). Finally the IgG is washed with an

ammonium sulfate solution ($40\%_{v/v}$) solution and stored in a refrigerator for an indefinite period.

Before further use, the IgG solution is centrifuged and the supernatant removed, the pellet is dissolved in a small amount of phosphate buffered saline, and dialysed against phosphate buffered saline to remove the ammonium sulfate. The concentration of IgG is determined spectrophotometrically using Equation 2.1:[158b]

$$c = d\,[2\,(A_{280}) - A_{260}] \qquad \text{Equation 2.1}$$

c is the concentration in mg/ml, d is the dilution factor and A is the absorbance at the respective wavelengths.

2.5 Routine Analyses

2.5.1 NMR Spectra

^{1}H NMR spectra were recorded either on a Brüker AC 200 (200 MHz) or on a Brüker AMX (400 MHz) spectrometer. Multiplicities are recorded as broad peaks (br), singlets (s), doublets (d), triplets (t), quartets (q) and multiplets (m).

2.5.2 Mass Spectra

Electron impact mass spectra were recorded on a VG micromass 7070E.

2.5.3 Melting Point

Melting points (m.p.) were determined on a Kofler block, are uncorrected, and expressed in degrees Celcius (°C).

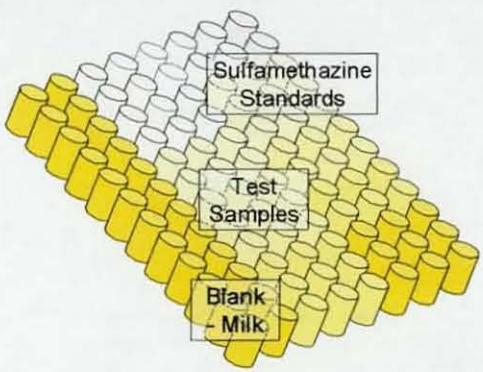
2.5.4 Enzyme Linked Immunosorbent Assays (ELISA)

Sheep anti-sulfamethazine antisera (21.5 mg/ml IgG, 10 μl) is dissolved in water (100 ml) and allowed to equilibrate to room temperature. 250 μl of the antisera solution is placed in each well of a 96 well microtitre plate and incubated in the refrigerator overnight.

A solution of sulfamethazine is prepared by adding 40 μl of standard sulfamethazine solution (10 μg/ml) into pasteurised semi-skimmed milk (3.960 ml) and a series of twelve sulfamethazine standard solutions are prepared by serial dilution. Similarly, a series of test compound standards are prepared.

The microtitre plate is washed with Tween-20 solution (500 ml) and the excess Tween-20 removed by vigorous shaking. The sulfamethazine standards (200 μl), test compound standards (200 μl) and the semi-skimmed milk blank (200 μl) are pipetted onto the plate (Figure 2.1).

Figure 2.1 Representation of 96 Well Microtitre Plate



Horseradish peroxidase/carmazine red solution (50 μl. 0.5 μg/ml) is added to the wells and the plate incubated at room temperature for 15 minutes with shaking. The plate is washed with Tween-20 (500 ml) and the excess Tween is removed by shaking. 3,3',5,5'-tetramethylbenzadine chromogenic substrate solution (200 μl) is added and the plate is incubated at room temperature for 15 minutes. The reaction is stopped by the addition of sulfuric acid solution (50 μl, 10%$_{v/v}$) and the plate is read using a TitreTek microtitre plate reader at 450 nm (reference 690 nm).[159,160]

2.5.5 Estimation of Sulfamethazine Binding Efficiency

The binding efficiency is an estimation of the antibody affinity for the conjugate, compared with that for sulfamethazine, and is determined by an ELISA assay using a horseradish peroxidase conjugate. The concentration of sample that gives fifty-percent antibody binding is used as an indication of the binding efficiency.

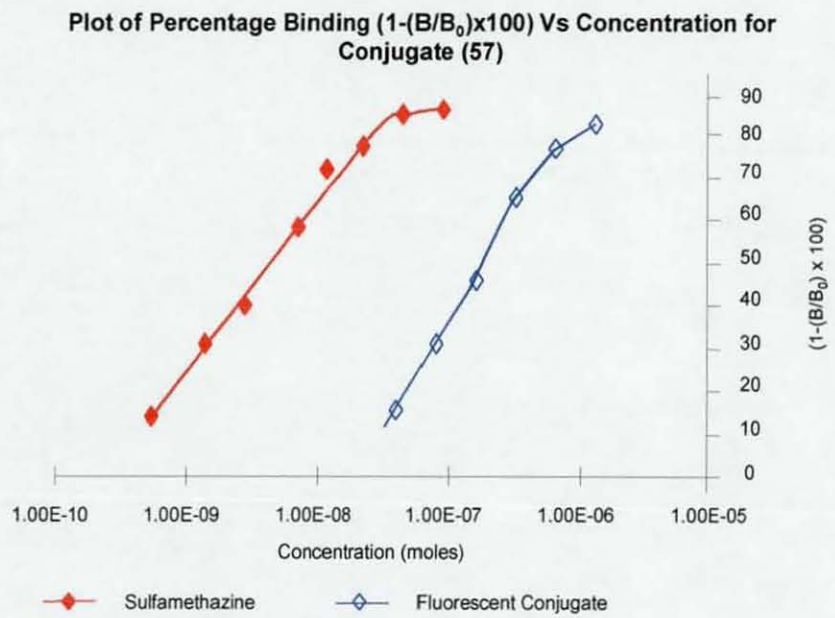
The percentage binding of the sample is calculated using Equation 2.2:

$$(1 - (B/B_0)) \times 100 \qquad \text{Equation 2.2}$$

B_0 is the mean of all the blank wells. It arises solely from the binding of horseradish peroxidase conjugate to the antibodies thereby giving the maximum signal for the plate. B is the mean obtained for the sulfamethazine standard and test compound at each concentration.

A plot of sample concentration versus percentage binding is used as an indication of the binding efficiency for the sample and is compared to the antibody binding of free sulfamethazine (Figure 2.2)

Figure 2.2 Representation of Conjugate Binding Efficiency



2.5.6 *Fluorolink™ Immunoassay*[161]

The antibody to be conjugated (1 mg) is dissolved in sodium carbonate buffer (1 ml), and added to the dye (5 mg). These are mixed thoroughly, taking care to prevent foaming, and incubated at room temperature for 30 minutes with additional shaking every 10 minutes.

The labelled protein is purified using an SG-50 gel filtration column with phosphate buffered saline (0.1 M, 0.9%$_{w/v}$, pH 7.4). Two bands should develop during elution, the faster moving band is the dye-labelled antibody while the slower band is free dye. The purified labelled protein can be stored at 4°C without further manipulation.

2.5.7 *Determination of Dye : Protein Ratio*

A portion of the labelled protein solution is diluted so that the maximum UV-Visible absorbance is 0.5 to 1.5. The molar concentrations of the dye, protein and the molar dye/protein ratio are calculated as outlined below (Equations 2.3 – 2.5).

In this example the molar absorption coefficients of 200,000 $M^{-1}cm^{-1}$ at 650 nm for cyanine dye (Cy5.18.OH, **(35)**) and 170,000 $M^{-1}cm^{-1}$ at 280 nm for antibody protein are used, and the calculation is corrected for the absorbance of the dye at 280 nm (approximately 5% of the absorbance at 650 nm).

$$[\text{dye}] = (A_{650})/200{,}000 \qquad \text{Equation 2. 3}$$

$$[\text{antibody}] = (A_{280} - 0.05 \times A_{650})/170{,}000 \qquad \text{Equation 2. 4}$$

$$(\text{dye/protein})_{final} = [\text{dye}]/[\text{antibody}] \qquad \text{Equation 2. 5}$$

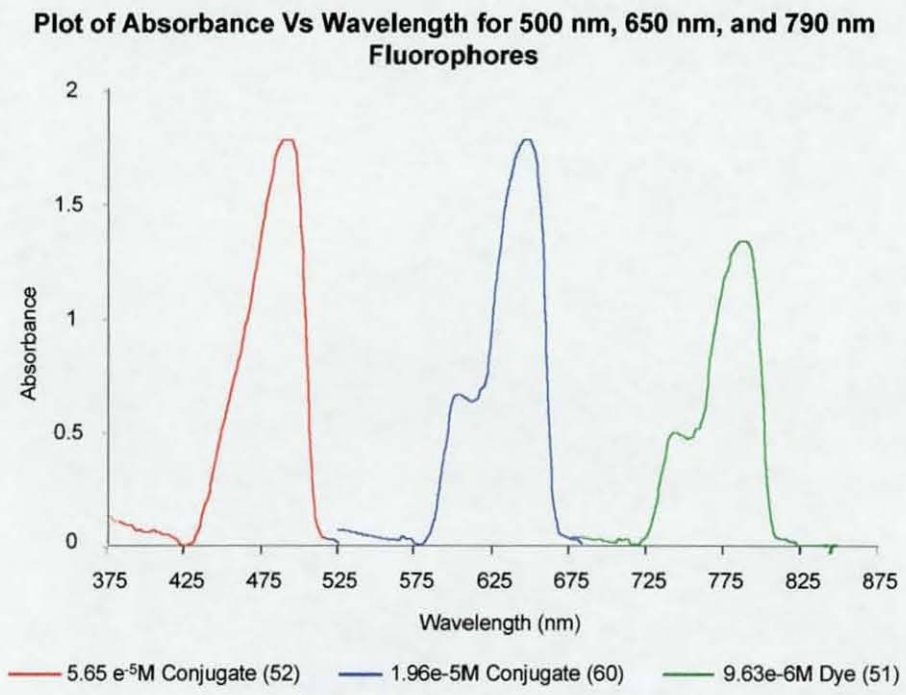
The molar absorption coefficient may vary for different proteins. For calculations involving horseradish peroxidase the absorbance occurs at 460 nm and has a molar absorption coefficient of 94,000 $M^{-1}cm^{-1}$.

2.5.8 *U.V-visible spectroscopy*

UV-Visible spectrophotometry was performed on a Philips PU8720 scanning spectrophotometer using quartz-silica cuvettes. It is mainly used to determine the λ_{max} and to calculate ε for the fluorophores, and to check the concentration of the conjugate solutions.

The instrument is blanked against the solvent being used and a scan is run between 190 nm and 900 nm (Figure 2.3).

Figure 2.3 UV-Visible Absorbance Spectra of 500 nm, 650 nm and 790 nm Fluorophores



The position of the maximum peak, λ_{max}, is used as the excitation wavelength for the sample and is used in the fluorimeter experiments.

2.5.9 *Determination of Molar Absorption Coefficient (ε)*

To determine ε the values obtained from a UV-Visible spectrum of a sample with known concentration are entered into Equation 2.6.

$$A = \varepsilon bc \qquad \text{Equation 2.6}$$

A is the absorbance at λ_{max}, b is the concentration in $molL^{-1}$, c is the path length in cm and ε is the molar absorption coefficient in $M^{-1}cm^{-1}$.

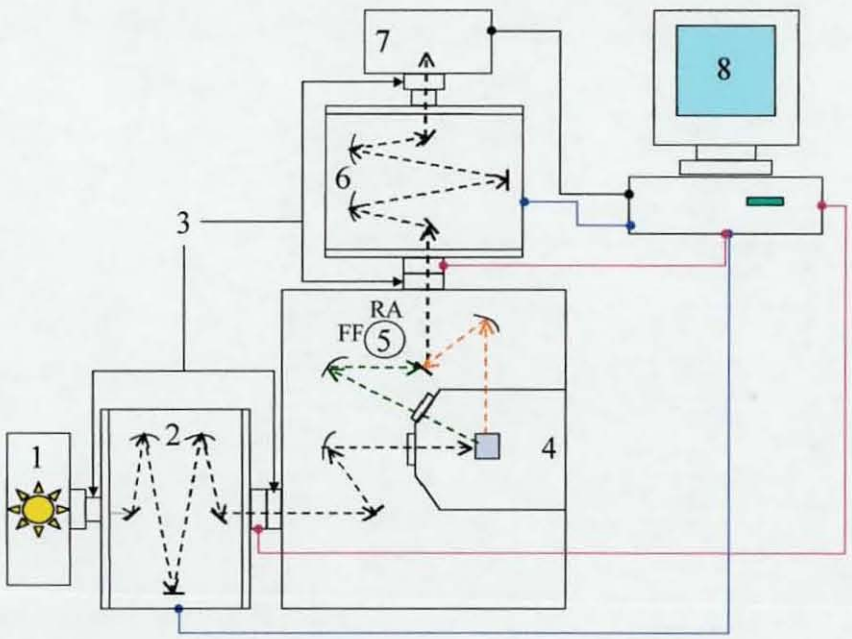
The concentration (in moles) is plotted against absorbance to generate a linear graph that passes through the origin. The gradient of the slope is the molar absorption coefficient for the compound expressed as $M^{-1}cm^{-1}$.

2.6 Instrumentation

2.6.1 Spex Fluorolog-2

The fluorescence spectroscopy is acquired using a single beam, research-grade, Spex Fluorolog-2 fluorimeter (Figure 2.4), supplied by Instruments SA (Plymouth, UK).

Figure 2.4 Arrangement of Spex™ Fluorolog-2 Fluorimeter



1: Light Source, 2: Excitation Monochromator, 3: Slits, 4: Sample Chamber, 5: Beam Selector Switch, 6: Emission Monochromator, 7: Photomultiplier Tube, 8: Computer

The light source of the Spex Fluorolog-2 is a Xenon arc lamp that is coupled to a lens, which focuses the single beam of light through the entrance slits (3) into the monochromator (2). The general-purpose 1681B Minimate-2 monochromators are used for controlling the excitation (2) and emission (6) wavelengths. This is achieved by focusing the incoming light onto an angled grating that divides the light into specific wavelengths. The light is directed into and out of the monochromator by mirrors.

Four slits (3) are used in the Spex Fluorolog-2 and are positioned at either side of the monochromators. These are adjusted by using 1679F straight slits, which slide into place at the entrance and exit ports of the Minimate-2. The slit width controls the bandpass of the emerging light, and the relationships between the slit width and bandpass are given in Table 2.2

Table 2. 2 Relationship of Slit Width and Bandpass

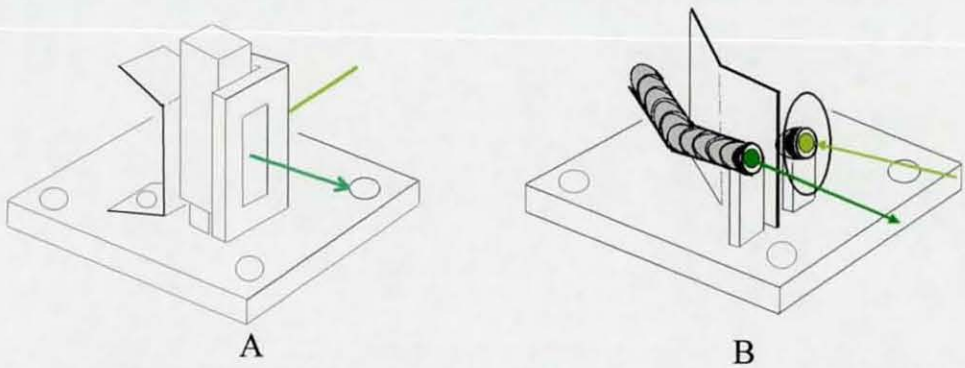
Slit Width (mm)	Bandpass (nm)
0.25	0.9
0.5	1.8
1.25	4.5
2.5	9.0
5.0	18
T (circular)	18

The sample chamber (4) is fitted with a light-tight lid that actuates the shutter control, which prevents room light from reaching the detector when the lid is opened. On the top of the chamber is the optical path selection switch (5), which controls the path taken by the light from the sample to the detector. Right angle (RA) collects radiation at a 90° angle from the excitation beam and is normally selected for viewing fluorescence in liquids. Front face (FF) collects radiation at an angle of 22.5° from the excitation beam and is useful for analysing solids and opaque samples.

The fluorescence intensity is detected using a standard side window photomultiplier tube (Spex 1411M) and the whole system is controlled by a 486 microprocessor installed with Spex software.

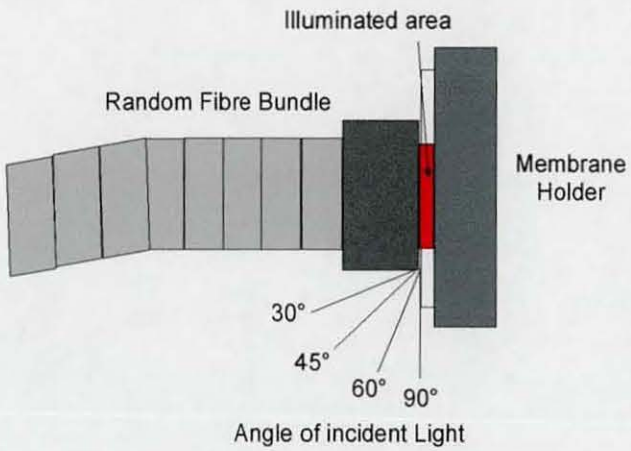
Two types of adapter are used in the Spex Fluorolog-2 these are a standard cuvette holder A (Figure 2.5), which is used for solution based fluorescence; and holder B, which is used for keeping bifurcated glass optical fibres (Oriel, USA) in place for remote fluorescence spectroscopy on surfaces.

Figure 2. 5 Adapters for Spex™ Fluorolog-2 Fluorimeter



When the fluorescence spectrum of the solid phase test strips is required a specially designed test strip holder is used (Figure 2.6). This clamps the membrane flush against the fibre end (90°) or allows the membrane to fibre angle to be varied.

Figure 2. 6 Test Strip Holder Indicating Angles of Detection



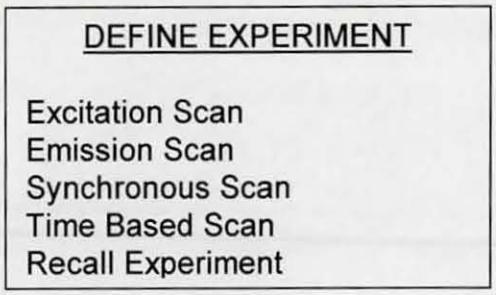
2.6.2 Operation of Fluorimeter

Due to the unique software employed to operate the Spex Fluorolog-2, the following section outlines the procedures used to obtain a spectrum.
When switching on the xenon arc lamp it is important that other electrical instruments that are on the same power circuit are switched off before sparking the lamp. Switch on the lamp power supply and ensure that the cooling fan is on before the lamp is ignited, then press **START** and wait for the lamp to ignite. It is now safe to switch on all other power supplies. Turn on the computer and type: **SYSTEM**, the following screens appear.

Select System to Run: Fluorescence DOS	Highlight Fluorescence Measurement Press Return ↵
Copyright 1986 – 1991 Spex Excitation Mono [ ] nm Emission Mono [ ] nm	Enter Excitation Monochromator value Press Return ↵ Enter Emission Monochromator value Press Return ↵
MAIN MENU Define Experiment Run Experiment Display Data Process Data Create Hard Copy Manage Files Program Keystrokes Set Defaults Other Activities Exit to System Shell to System	Press **F4** to bring up the Main Menu

To switch on photomultiplier tube by pressing **F10**

HIGH VOLTAGE HV1 [950] Status [OFF] Actual HV is 0 GO	Press Return ↵ then **Space** This turns Status from **OFF** to **ON** Press Return ↵ twice This returns you to the Main Menu



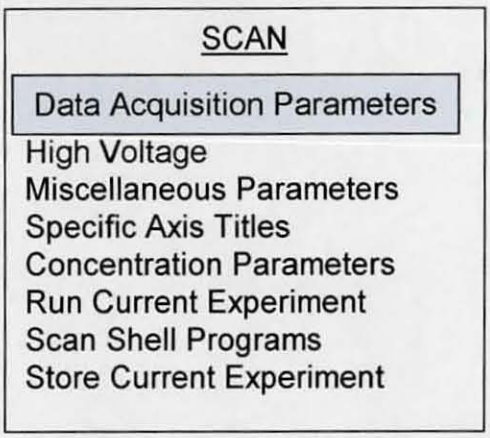
Using ↕↔ Keys

Highlight **Define Experiment**

Press Return ↵

Select type of scan required

Press Return ↵

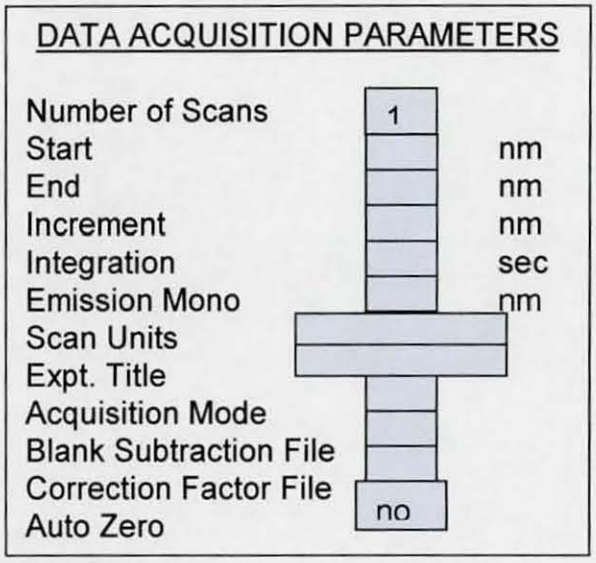


Highlight **Data Acquisition Parameters**

Press Return ↵

Put in the relevant parameters

After each entry Press Return ↵



When all parameters are correct

Press Return ↵

And return to main menu (**F4**)

Highlight **Run Experiment**

Input **Data File** name

Press Return ↵

Input **Data Title** (optional)

Press Return ↵

The fluorimeter will perform the scan and display the spectrum on the monitor. Return to the main menu to print the spectrum, highlighting **Create Hard Copy** (press Return ↵). To display more than one spectrum select **Display Data**, to label data and convert data files to ASCII files for use with other applications use **Other Activities**. To shut down the fluorimeter select **Exit To System**, switch off the computer, press **Stop** on the lamp and allow 5 minutes cooling time before switching off the power.

2.6.3 Calibration of Spex Fluorolog-2

The Spex Fluorolog-2 is calibrated by running a Raman spectrum of water (Appendix 3, Figure A.3.1). Set the beam selector knob to RA and insert 1.25 mm slits all round. Place a water-filled cuvette into the sample holder A and close the lid. Run an emission scan as outlined in Section 2.6.2 using the following parameters in Table 2.3.

Table 2. 3 Fluorimeter Calibration Parameters

Excitation	350 nm	**Emission**	365 – 450 nm	**Increment**	1 nm
Integration	0.5 s	**Slits – Excitation**		1.25, 1.25	
		Emission		1.25, 1.25	

If the water Raman spectrum is not satisfactory, the lamp may need refocusing or replacing, or the PMT replacing.

2.6.4 Importing Data to Excel

Once the ASCII file has been created by the fluorimeter select, **Shell To System** (Return ↵),the files are saved in directory DM3000F, open the directory type

C: \> **CD DM3000F** (Return ↵)

to copy data files to a floppy disc type

C : \>DM3000F>**Copy {file name}.TXT A:** (Return ↵)

The files will be copied onto a floppy disc for importing into Excel or other applications.

Open Excel and in the file menu select OPEN. Select $3^1/_2$ floppy drive A: and select files for importing. Follow the menu instructions given in Excel for importing the data.

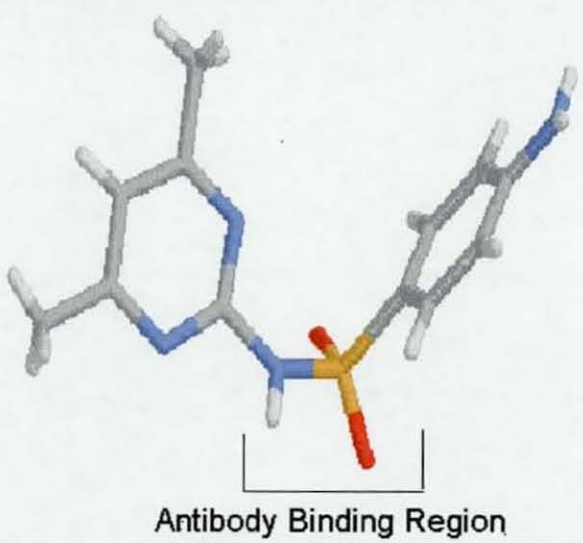
CHAPTER THREE

SYNTHESIS OF FLUORESCENT DYE CONJUGATES

3.1 Introduction

The aim of the synthesis was to fluorescently label sulfamethazine without altering its antibody-binding region (Figure 3.1) or affecting the fluorescence characteristics of the label. The approaches taken for the labelling sulfamethazine was to indirectly label sulfamethazine to a fluorophore using a protein as a linker group, or directly label using a chemical spacer group. It was thought that adding a spacer group would enable a sufficient distance between the fluorophore and sulfamethazine to be attained, which would reduce any electron withdrawing effect on the fluorophore by the sulfamethazine and prevent steric hindrance of the antibody binding region. The spacer groups chosen were long aliphatic chains.[162]

Figure 3. 1 Representation of "Elbow" Shaped Binding Region of Sulfamethazine[163,20]



The fluorophores were chosen primarily for the wavelength at which they fluoresce. The dyes at 490 nm and 650 nm offered a ready starting point as they were commercially available and seemed easy to make. The purification of the dyes was known to be difficult because of their limited

solubility and photo-instability; it was anticipated that the purification problem would become of great importance when synthesising the more complicated dyes in the 750 nm region, as these dyes have been reported to be less photo-stable than the 650 nm dyes. To overcome the photo-stability problem a stabilising group was incorporated into the methine bridge of the heptamethine cyanine dyes prepared in later syntheses.

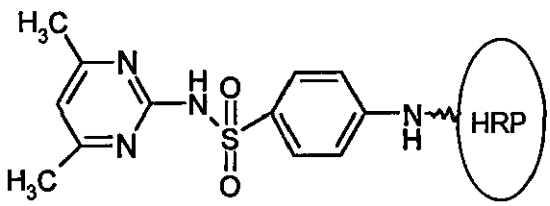
A decrease in the fluorescence characteristics on linking to sulfamethazine was anticipated but this had been allowed for by choosing fluorophores with high molar absorbances and relatively large quantum efficiencies. As the fluorescence was to be read off a solid surface a large Stokes shift was required to reduce the interference from the incident light reflected off the surface of the immunoassay test strip. Therefore conjugates of fluorescein isothiocyanate **(34)**, Cy5.18.OH **(35)**, Cy7.18.OH **(36)** and a heptamethine cyanine dye **(51)** were synthesised (Figure 3.2).

Figure 3.2 Photograph of Conjugates (52), (60), and (61)

3.2 Spacing Groups For Sulfamethazine

The antibodies used in the ELISA assay were provided by Roy Jackman and were produced using an immunogen linked via the primary amine group of sulfamethazine. Therefore an aromatic primary amino group was used for the linking process, as this would allow the immunogenic determinant (sulfonamide group) to be recognised by the antibodies.
Jackman *et al.* found that a spacer of 8 carbons was sufficient to prevent steric hindrance of the sulfonamide region, thus long aliphatic chains with an active group or activatable group at the terminal carbon to facilitate dye conjugation were synthesised.[75]

3.2.1 Preparation of Sulfamethazine-Horseradish Peroxidase



(37)

3.2.1.1 Method One[164]

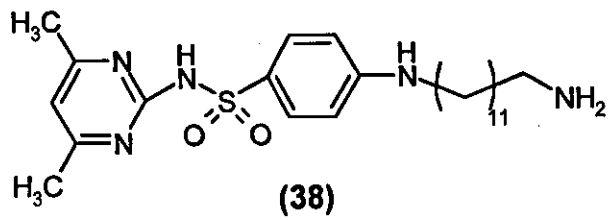
A solution of horseradish peroxidase (HRP), (100 mg) and sodium periodate (5.0 ml, 0.1 M) in water (12 ml) was stirred for 20 minutes at room temperature and the fractions were tested for excess oxidant. A solution of 1,2-diaminoethane (340 μl) in sodium carbonate buffer (8 ml, pH 9.5) was prepared and 0.7 ml was added to the HRP solution. The pH was lowered to 9.5 using saturated sodium hydrogen carbonate, the solution was stirred for 2 hours at room temperature, and passed through an SG-50 gel filtration column (water elution, 3.5 ml/min). Sodium borohydride (18 mg, 0.50 mol) was added to the HRP fractions and left at 4°C overnight. The solution was columned (as above), and the HRP

fractions were dialysed against polyethyleneglycol (20,000) for 2 hours. The concentration of activated HRP was determined spectrophotometrically (406 nm) and the solution chilled to less than 4°C. A solution of sulfamethazine **(1)** (21 mg, 0.0755 mmol) in hydrochloric acid (20 ml, 0.1 M) was chilled to less than 4°C. Sodium nitrite (0.15 M) was added in 0.1 ml aliquots until the oxidant was in excess, maintaining the temperature below 4°C. The solution of diazotised sulfamethazine was added to the activated HRP in a 4:1 molar ratio, stirred for 1 hour at room temperature. The solution was columned (as above), the HRP-sulfamethazine conjugate **(37A)** was collected and the concentration was determined spectrophotometrically (calculated as $A_{(406\ nm)}$/1.6 gave mg/ml).

3.2.1.2 Method Two[29]

Horseradish peroxidase (20 mg) and *p*-benzoquinone (200 μl, 0.15 mmol) were dissolved in phosphate buffered saline (2 ml, 0.01 M, pH 6.0) and stirred for 2 hours. The solution was dialysed against sodium chloride solution (0.9%$_{w/v}$, 2 x 5 L) for 4 hours. The dialysed peroxidase solution was added to a solution of sulfamethazine **(1)** (20 mg, in 1.6 ml PBS : 400 μl DMF) and adjusted to pH 8.0 by the addition of sodium carbonate buffer (0.1 M, pH 9.6). The solution was incubated at 4°C for 36 hours without stirring. Lysine (400 μl, 1 M) was added and the solution stirred at room temperature for 2 hours followed by dialysis against phosphate buffered saline solution (3 x 5 L, pH 7.4). The product of this reaction was denoted conjugate **(37B)** and the concentration was determined as outlined in 3.2.1.1.

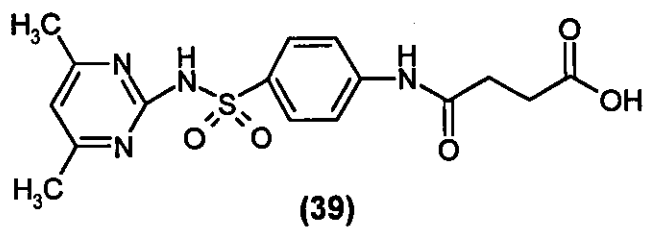
3.2.2 *Preparation of N1-4,6-dimethyl-2-pyrimidinyl-4-[(12-amino dodecyl)amino]-1-benzene sulfonamide*[164]



(38)

A solution of sulfamethazine **(1)** (500 mg, 1.80 mmol) in hydrochloric acid (50 ml, 1 M) was stirred at 1°C. Sodium nitrite solution (0.15 M) was added in 0.1 ml aliquots until in excess. 1,12-diaminododecane (550 mg, 2.75 mmol) was suspended in pH 9.4 carbonate buffer (50 ml, 1 M), stirring vigorously, and the diazotised sulfamethazine (15 ml) was added in 0.5 ml aliquots over a 90 minute period. A peach coloured precipitate formed. The solid was filtered under vacuum, washed with water followed by carbonate buffer (pH 9.4) and re-suspended in 40% methanol/60% ethyl acetate. The product was filtered, dried in a vacuum oven at 50°C and used in further reaction as an impure solid. $C_{24}H_{39}N_5SO_2$; TLC (60% methanol/40% ethyl acetate): R_f = 0.1; Yield 26%; m.p. = 163°C

3.2.3 *Preparation of 4-(4-{[4,6-dimethyl-2-pyrimidinyl)amino] sulfonyl}anilino)-4-oxo butanoic acid*[75]

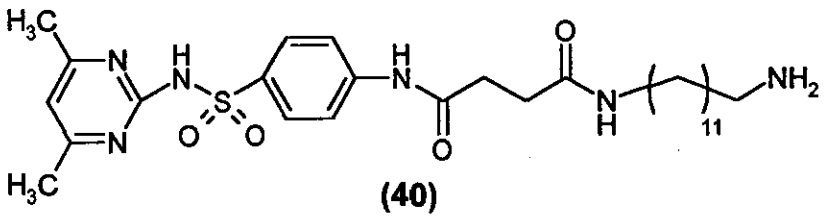


(39)

A solution of sulfamethazine **(1)** (3.03 g, 10.0 mmol) and succinic anhydride (1.51 g, 15.0 mmol) in ethanol (30 ml) was refluxed for 90 minutes. The solution was left to cool overnight and white crystals

was decanted off and the solid refluxed in a mixture of water/ethanol (1 : 1.5, 10 ml) for 10 minutes. The solution was cooled, filtered, washed three times in water/ethanol (1 : 1.5, 10 ml) and dried in a vacuum oven at 45°C. $C_{16}H_{18}N_4O_5S$; RP_{C-18} TLC (100% methanol): R_f = 0.87; Yield 85%; m.p. = 242°C; Mass Spec.: M^+ = 378.4; ^1H NMR *(200 MHz, MeOD-d_4)*: δ 7.17 (d, 2H, *J* = 14 Hz), 6.92 (d, 2H, *J* =14 Hz), 5.90 (s, 1H), 2.51 (t, 2H, *J* = 7 Hz), 1.80 (t, 2H, *J* = 7 Hz), 1.50 (s, 6H). The exchangeable hydrogens were not seen.

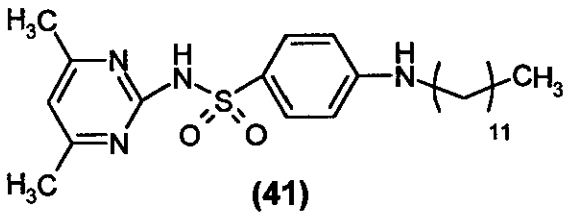
3.2.6 Preparation of N1-(12-aminododecyl)-N4-(4-{[(4,6-dimethyl-2-pyrimidinyl) amino]sulfonyl}phenyl)succinimide[111]



(40)

4-(4-{[4,6-dimethyl-2-pyrimidinyl)amino]sulfonyl}anilino)-4-oxobutanoic acid **(39)** (100 mg, 0.37 mmol), 1,12-diaminododecane (1 g, 5 mmol) and 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) (20 mg) were suspended in de-ionised water (15 ml) and methanol (2 ml) and was stirred overnight. A white precipitate formed and was centrifuged at 7000 rpm for 30 minutes. The pellet was re-dissolved in methanol, filtered and the filtrate evaporated to dryness. Water (2 ml) was added, the solid re-centrifuged and dried in a vacuum oven at 60°C. The pellet was recyrstallised from methanol. $C_{28}H_{44}N_6O_4S$; TLC (100% methanol) R_f = 0.85; Yield 89%. This was used crude in subsequent reactions.

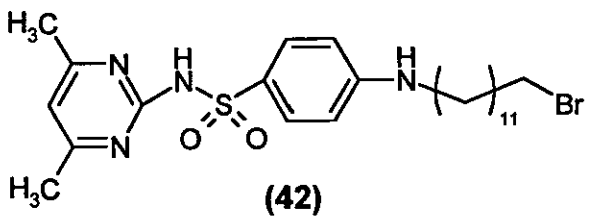
3.2.7 *Preparation of N1-(4,6-dimethyl-2-pyrimidinyl)-4-[(dodecyl) amino]-1-benzene sulfonamide*[165]



(41)

1-bromododecane (0.85 ml, 3.28 mmol) was added in 0.085 ml aliquots over a 30 minute period to a solution of sulfamethazine **(1)** (1.848 g, 6.65 mmol) in water (12 ml) and sodium hydrogen carbonate (12 g). The solution was refluxed at 95°C for 3 hours, cooled and filtered. Concentrated hydrochloric acid (31%) was added dropwise until pH 7.4 was attained. A peach coloured precipitate was formed, which was filtered and dried under vacuum. $C_{24}H_{38}N_4O_2S$; TLC (100% methanol): R_f = 0.69; Yield 85%; m.p. = 190°C; Mass Spec.: M^+ 446.27; ^1H NMR *(200 MHz, MeOD-d_4)*: δ 7.00 (d, 2H), 5.80 - 5.50 (d, 2H; s, 1H), 4.00 (s, 2H, NH), 1.25 (s, 6H), 0.75 - 0.25 (m, 25H) .

3.2.8 *Preparation of N1-(4,6-dimethyl-2-pyrimidinyl)-4-[(12-bromododecyl)amino]-1-benzene sulfonamide*[165]

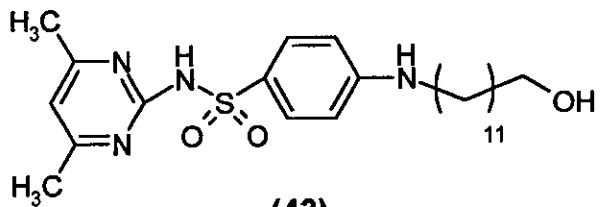


(42)

A solution of N1-(4,6-dimethyl-2-pyrimidinyl)-4-[(dodecyl)amino]-1-benzene sulfonamide **(41)**, (0.545 g, 1 mmol) and N-bromosuccinimide (0.178 g, 1 mmol) in chloroform (40 ml) was boiled for 180 min. The reaction was cooled, and the solvent removed *in vacuo*. A brown waxy

solid formed. This was resuspended in methanol, filtered and the solvent removed. $C_{24}H_{37}N_4O_2SBr$; TLC (100% methanol): R_f = 0.67; Yield 70%; m.p. = 191°C; ^{1}H NMR *(200 MHz, MeOD-d_4)*: δ 7.90 (d, 2H), 7.27 (s, 1H), 6.65 (d, 2H), 4.72 (s, 2H, NH), 3.67 - 3.76 (m, 2H), 3.36 - 3.53 (m, 2H), 2.37 (s, 6H), 2.29 - 2.49 (m, 2H), 1.73 - 1.91 (m, 2H), 1.23 - 1.45 (m, 16H)

3.2.9 Preparation of N1-(4,6-dimethyl-2-pyrimidinyl)-4-[12-hydroxy dodecyl)amino]-1-benzene sulfonamide

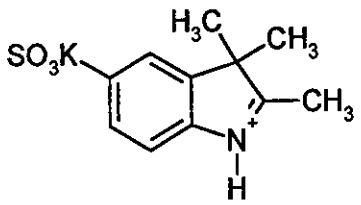


(43)

Sulfamethazine **(1)** (0.2098 g, 0.754 mmol) and triethylamine (0.0763 g, 0.754 mmol) were dissolved in THF (3 ml) with stirring. 12-bromo-1-dodecanol (0.2 g, 0.754 mmol) was dissolved in THF (0.5 ml) and added dropwise in 0.05 ml aliquots over a 1 hour period, then stirred at room temperature overnight. The mixture was filtered and the filtrate dried. TLC (80% ethyl acetate/20% methanol/1% acetic acid): R_f = 0.95; Yield 23%; ^{1}H NMR *(200 MHz, MeOD-d_4)*: δ 7.70 (d, 2H), 6.70 (d, 2H, s, 1H), 4.90 (s, 3H, OH, NH), 3.60 (t, 2H), 3.40 (t, 2H), 2.30 (s, 6H), 1.70 - 1.90 (m, 2H), 1.20 - 1.50 (m, 18H).

3.3 Fluorescent Dye Synthesis

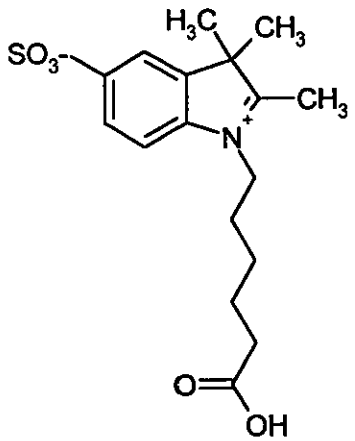
3.3.1 Preparation of 2,3,3-Trimethylindoleninium-5-sulfonate[112]



(44)

A solution of 3-methyl-2-butanone (84 ml, 0.784 mol) in acetic acid (150 ml) was added to *p*-hydrazinobenzenesulphonic acid (50 g, 0.27 mol) and refluxed for 3 hours. The reaction mixture was cooled and a pink solid formed on trituration. The solid was separated under vacuum and redissolved in a minimal amount of methanol. Addition of 2-propanol saturated with potassium hydroxide gave the yellow potassium salt as a precipitate. The solid was filtered, recrystallised with 2-propanol/KOH and dried in an oven at 45°C. $C_{11}H_{14}NSO_3K$; TLC (100% methanol): R_f 0.75; Yield 33%; m.p. = 293°C; Mass Spec.: No M^+ ion at 278, fragments at 159, 121, 119 and 103, which was consistent with literature findings. The potassium salt was used in further reactions as an impure solid.

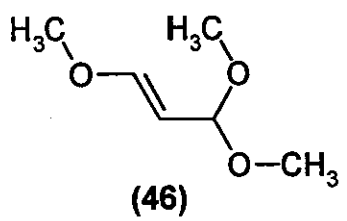
3.3.2 *Preparation of 1-(carboxypentynyl)-2,3,3-trimethyl indoleninium-5-sulfonate*[104]



(45)

A solution of the potassium salt of 2,3,3-trimethylindoleninium-5-sulfonate **(44)**, (1.1 g, 3.95 mmol), in 1,2-dichlorobenzene (10 ml) was added to bromohexanoic acid (0.981 g, 5.02 mmol) and refluxed for 12 hours under nitrogen. The mixture was cooled, the 1,2-dichlorobenzene decanted, 2-propanol (35 ml) was added and the solid filtered. The solid was washed twice in 2-propanol and used in further reactions to make Cy5.18.OH **(35)** and Cy7.18.OH **(36)** as an impure solid. $C_{17}H_{24}NSO_5$; TLC (100% methanol): R_f = 0.95; m.p. = 295°C (Lit Value 295°C)

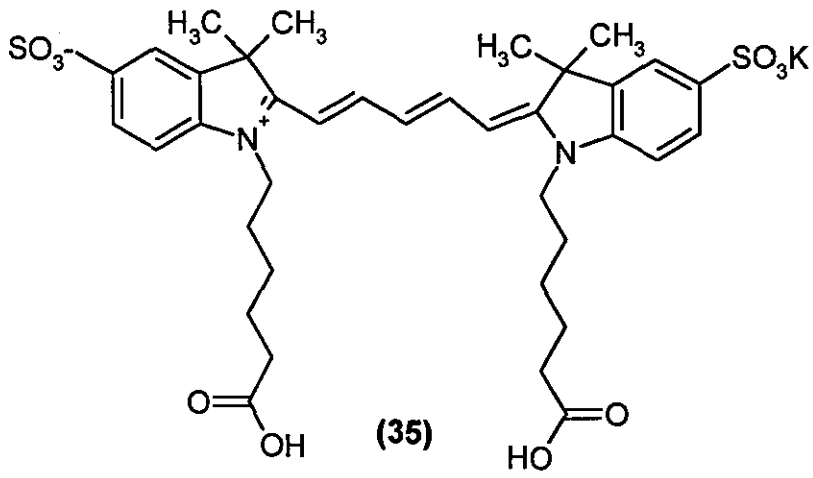
3.3.3 *Preparation of 1,3,3-trimethoxypropene*[166]



(46)

1,3,3-trimethoxypropene was made according to the procedure of Lounasmaa *et al.* and the structure confirmed by comparison with an authentic sample supplied by Avocado Ltd.

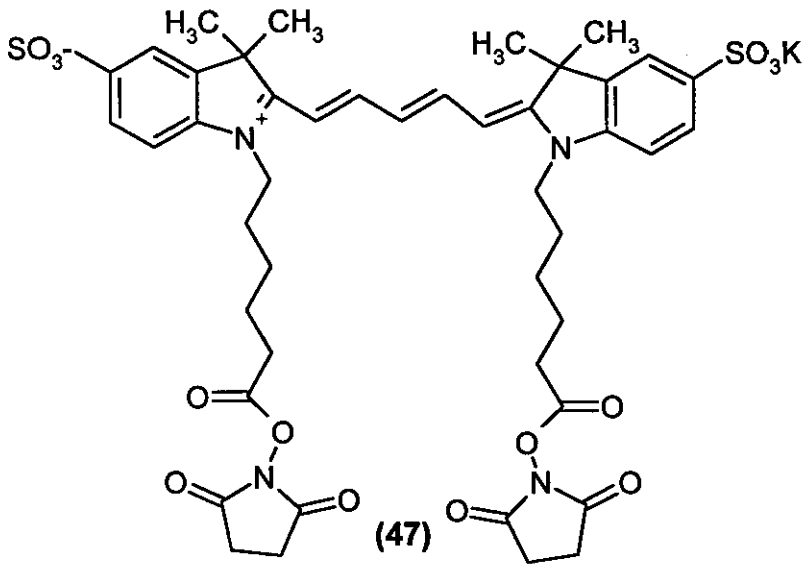
3.3.4 *Preparation of 6-[2-{(1E,3E)-5-[1-(5-carboxypentyl)-5-(hydroxy sulfonyl)-3,3-dimethyl-2,3-dihydro-1H-2-indolyliden]-1,3-[pentadienyl}-5-hydroxysulfonyl)-3,3-dimethyl-3H-1-indoliumyl] hexanoic acid.*[132] (Cy5.18.OH™)



(35)

A solution of 1-(carboxypentynyl)-2,3,3-trimethylindoleninium-5-sulfonate **(45)** (4.99 g, 0.014 mol) in methanol (30 ml) was added to potassium acetate (0.71 g, 7 mmol) and refluxed at 60°C for 180 minutes. 1,3,3-trimethoxypropene **(46)** (1 ml) was added every 30 minutes until a total of 4 ml had been added. A thick blue precipitate formed. The solid was filtered and washed with minimal hot methanol. The product was re-precipitated from the filtrate with 2-propanol then filtered and dried. Preparative $RP_{C\text{-}18}TLC$ (60% methanol/40% water) reveals 3 bands R_f = 0.90 - 0.95. The band at R_f = 0.95 was scraped off, dissolved in methanol, re-precipitated in 2-propanol, filtered and dried. $C_{37}H_{45}O_{10}N_2S_2K$; Yield 21% (crude); λ_{max} = 649 nm; ε = 200,000 $M^{-1}cm^{-1}$; ^1H NMR *(400 MHz, DMSO-d_6)*: δ 7.9 (t, 2H, *J* = 14 Hz), 7.7 (s, 2H), 7.6 (d, 2H, *J* = 8 Hz), 7.4 (d, 2H, *J* = 7.6 Hz), 6.4 (t, 1H, *J* = 15 Hz), 6.0 (d, 2H, *J* = 15 Hz), 3.9 (t, 4H, *J* = 7.5 Hz), 2.15 (t, 4H, *J* = 7.5 Hz), 1.7 (m, 4H), 1.3-1.66 (m, 8H), 1.5 (s, 12H). The exchangeable hydrogens were not seen.

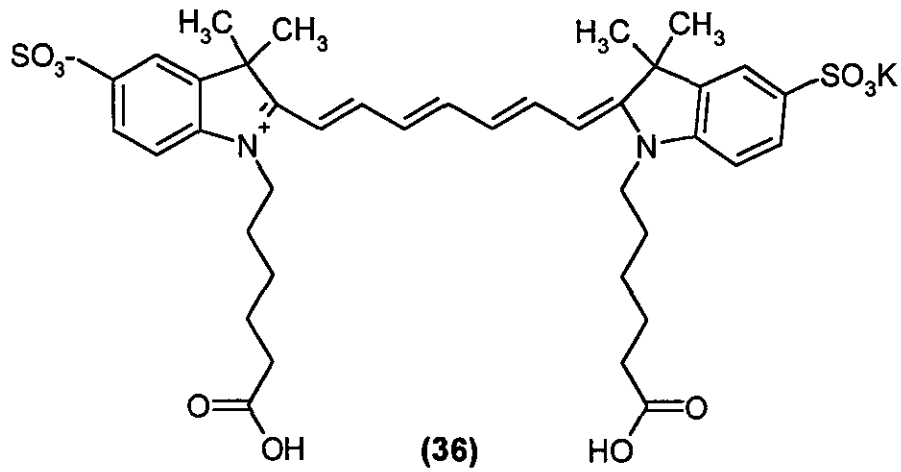
3.3.5 *Preparation of 6-[2-{(1E,3E)-5-[1-{(5carboxypentyl) succinimidyl}-5-(hydroxysulfonyl)-3,3-dimethyl-2,3-dihydro-1H-2-indolyliden]-1,3-pentaideyl}-5-hydroxysulfonyl)-3,3-dimethyl-3H-1-indoliumyl]hexanoyl succinimide*[131]



(47)

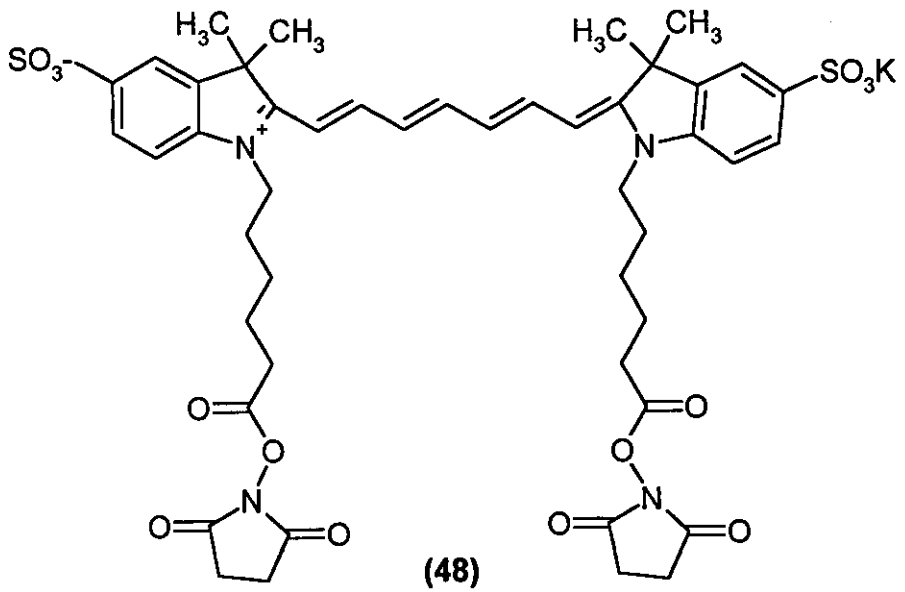
N,N'-disuccinimidyl carbonate (0.21 g, 0.807 mmol) was added to a solution of Cy5.18.OH **(35)** (200 mg, 0.269 mmol), in dry DMF (4 ml) and dry pyridine (0.2 ml) and heated with stirring to 55-58°C for 90 minutes under nitrogen. Dry ethyl acetate (10 ml) was added and immediately a blue precipitate formed. The solid was filtered, washed with dry ethyl acetate (4 x 10 ml) and dried in a vacuum oven at 55°C. $C_{45}H_{51}O_{14}N_4S_2K$; $RP_{C\text{-}18}$ TLC (60% methanol/40% water): R_f = 0.15; Yield 99%; λ_{max} = 652 nm. The product was tested by the Fluorolink assay for succinimidyl activity and found to give a 2 : 1 dye : protein ratio.

3.3.6 Preparation of 6-[2-{(1E, 3Z, 5Z)-7-[1-(5-carboxypentyl)-5-(hydroxysulfonyl)-3,3-dimethyl-2,3-dihydro-1H-2-indolyliden]-1,3,5-heptatrienyl}-5-(hydroxysulfonyl)-3,3-dimethyl-3H-1-indoliumyl]hexanoic acid (Cy7.18.OH™)



A solution of glutaconaldehyde dianil hydrochloride (500 mg, 1.75 mmol) in acetic anhydride (14 ml) and pyridine (3.5 ml) was heated to 45°C. 1-(carboxypentynyl)-2,3,3-trimethylindoleninium-5-sulfonate **(45)** (1.234 g, 3.486 mmol), was added and the solution refluxed at 120°C for 10 minutes. The solution was cooled to room temperature and diethylether (30 ml) added. A green precipitate formed. The ether was decanted and the precipitate was redissolved in a minimal amount of hot methanol. 2-propanol (30 ml) was added and a green/blue precipitate formed. The compound was recrystallised 3 times from methanol/2-propanol and dried in a vacuum oven at 50°C. $RP_{C\text{-}18}$ TLC (25% methanol/75% water) revealed a mixture of compounds the major band being at R_f = 0.30. The band at R_f = 0.30 was removed, dissolved in methanol, re-precipitated from 2-propanol, filtered and dried. $C_{39}H_{49}O_{10}N_2S_2K$; Yield 68%; λ_{max} = 765 nm; ε = 250,000 $M^{-1}cm^{-1}$

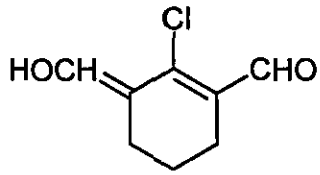
3.3.7 *Preparation of 6-[2-{[1E, 3Z, 5Z)-7-{1-((5-carboxypentyl)succinimidyl)-5-(hydroxysulfonyl)-3,3-dimethyl-2,3-dihydro-1H-2-indolyliden]-1,3,5-heptatrienyl}-5-(hydroxysulfonyl)-3,3-dimethyl-3H-1-indoliumyl]hexanoyl succinimide*



(48)

N,N'-disuccinimidyl carbonate (0.257 g, 0.987 mmol) was added to a solution of Cy7.18.OH **(36)** (387 mg, 0.503 mmol) in dry DMF (7.74 ml) and dry pyridine (0.38 ml) and heated with stirring to 55-58°C for 90 minutes under nitrogen. Dry ethyl acetate (10 ml) was added and immediately a green/black precipitate formed. The solid was filtered, washed with dry ethyl acetate (4 x 10 ml), and dried in a vacuum oven at 55°C. RP_{C-18} TLC (60% methanol/40% water): R_f = 0.15; Yield 36%; m.p. > 400°C; λ_{max} = 758.4 nm. Succinimidyl ester activity determined by Fluorolink method and found to give 7:1 dye:protein ratio.

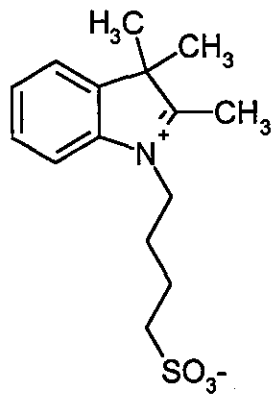
3.3.8 *Preparation of 2-chloro-3-[(E)-1-hydroxymethylidene]-cyclohexene-1-carbaldehyde*[167,168]



(49)

2-Chloro-3-[(E)-1-hydroxymethylidene]-cyclohexene-1-carbaldehyde was made according to the procedure of Reynolds and Drexhage.[141] The reaction was performed on a 5 mmol scale and yielded 54% product with a m.p. = 127°C (Lit value 130°C). The yellow solid was stable for 24 hours and was therefore used immediately in subsequent reactions.

3.3.9 *Preparation of 4-(2,3,3-trimethyl-3H-indoliumyl)-1-butanesulfonate*[141]

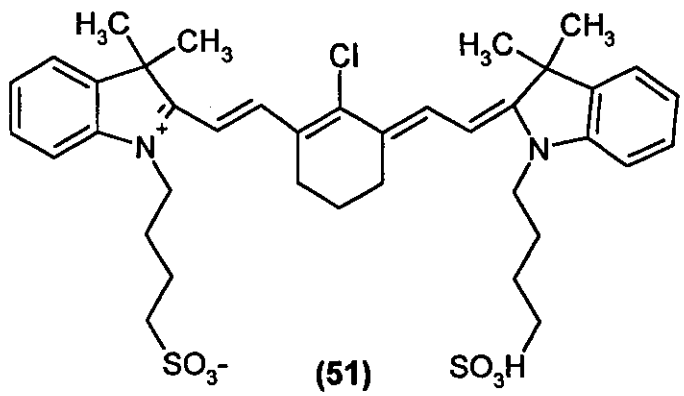


(50)

A solution of 2,3,3-trimethylindolenine (3.61 g, 0.0225 moles) in anhydrous 1,2-dichlorobenzene (50 ml) was added to 4-butane sultone (6.88 ml, 0.0676 moles) and refluxed at 120°C for 12 hours under nitrogen. A dark purple solid formed on cooling. The 1,2-dichlorobenzene was decanted, the solid was washed with acetone, purified by repeated crystallisation from acetone/methanol and dried in an oven at 50°C.

$C_{15}H_{21}O_3NS$; TLC (100% methanol): R_f = 0.60; Yield 34%; m.p. = 221°C; ^{1}H NMR *(200 MHz, DMSO-d_6)*: δ 8.03 (d, 1 H, 7.6 Hz), 7.82 (d, 1 H, 7.6 Hz), *7.59* - 7.63 (m, 2H), 4.45 (t, 2H, *J* = 7.6 Hz), 2.48 - 2.54 (t, 2H), 1.94 - 2.00 (m, 2H), 1.71 - 1.77 (m, 2H), 1.53 (s, 9H), 1.09 (t, 1H, *J* = 7.6 Hz)

3.3.10 Preparation of 4-[2-[4-chloro-7(3,3-dimethyl-1-(4-sulfonatobutyl)indolin-2-ylidene]-3,5-(propane-1,3-diyl)-l,3,5-heptatrien-1-yl]-3,3-dimethyl-3H-indolio]butane sulfonate[156]

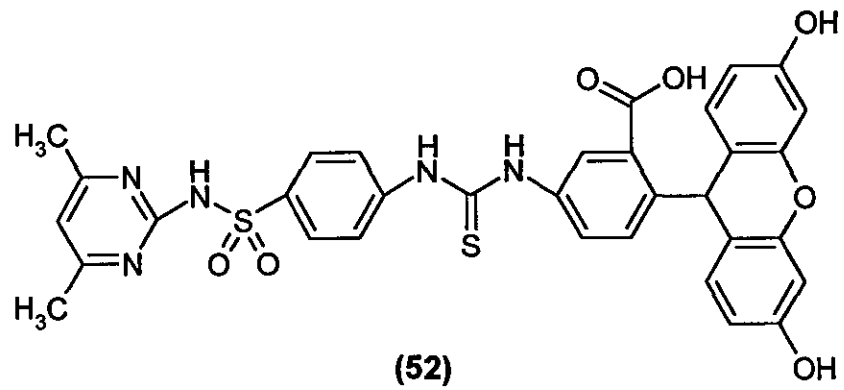


(51)

A solution of 4-(2,3,3-trimethyl-3H-indolio) butanesulfonate **(50)** (0.4431 g, 1.5 mmol) and 2-chloro-l-formyl-3-hydroxymethylene cyclohexene **(49)** (0.1294 g, 0. 75 mmol) in benzene/butan-1-ol (30 ml : 70 ml) was refluxed using a Dean-Stark condenser at 95°C for 11 hours. The solvents are removed *in vacuo* and the solid washed in diethyl ether. The solid was passed down an LH-20 gel chromatography column (eluting methanol, 1.0 ml/min) and the green fragments collected. $C_{38}H_{47}O_6N_2S_2Cl$; TLC (75% chloroform/25% methanol): R_f = 0.28; Yield 97% (Crude); m.p. = 242°C; λ_{max} = 783 nm; ε = 135,210 $M^{-1}cm^{-1}$; ^{1}H NMR *(400 MHz, DMSO-d_6)*: δ 8.14 (d, 2H, *J*= 14.0 Hz), 7.52 (d, 2H, *J* = 7.6 Hz), 7.28 - 7.41 (m, 4H), 7.16 (t, 2H, *J* = 7.6 Hz), 6.30 (d, 2H, *J* = 14.0 Hz), 4.16 (t, 4H, *J* = 6.8 Hz), 2.47 (t, 4H, *J* = 5.6Hz), 1.67 (s, 12H), 0.10 - 0.19 (m, 14H)

3.4 Fluorescent Dye Conjugate Synthesis

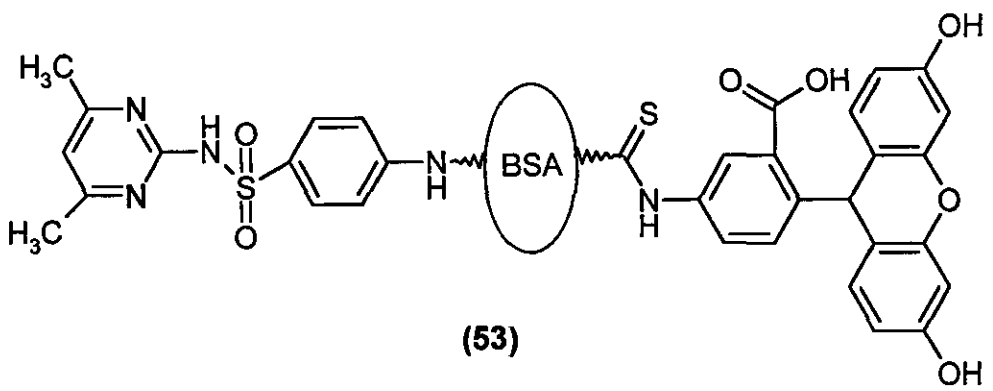
3.4.1 Preparation of N1-(4,6-dimethyl-2-pyrimidinyl)-4-{fluoresceln}-6-amino]carbothioyl}amino)-1-benzene sulfonamide[75]



(52)

Sulfamethazine **(1)** (10 mg, 36 μmol) was dissolved in a mixture of methanol (0.5 ml), triethylamine hydrochloride (49 mg, 0.36 mmol) and fluorescein isothiocyanate **(34)** (7 mg, 18 μmol) and stirred at room temperature for 16 hours. The conjugate was purified by preparative TLC (80% ethyl acetate/19% methanol/1% acetic acid), where the band at R_f = 0.65 was removed and extracted into methanol. $C_{33}H_{27}N_5O_7S_2$; Yield 42%; λ_{max} = 493 nm; ε = 32,086 $M^{-1}cm^{-1}$; FAB Mass Spec.: No molecular ion seen, fragments at 651, 593, 407, 273; ^{1}H NMR *(400MHz, DMSO-d_6)*: δ 8.54 (s, 1H), 8.05 (d, 2H, *J* = 7.6 Hz), 8.03 (d, 1H, *J* = 7.6 Hz), 7.81 - 7.84 (dd, 1H, *J* = 7.6 Hz), 7.75 (d, 2H, *J* = 14 Hz), 7.21 (d, 1H, *J* = 14 Hz), 7.08 (d, 2H, *J* = 14 Hz), 6.61 - 6.68 (m, 5H), 3.34 (s, 3H), 2.30 (s, 6H) the exchangeable hydrogens were not seen; ELISA assay $B/B_{0(50\%)}$ = $1e^{-7}$ M (cf. Sulfamethazine **(1)** = $6e^{-8}$ M)

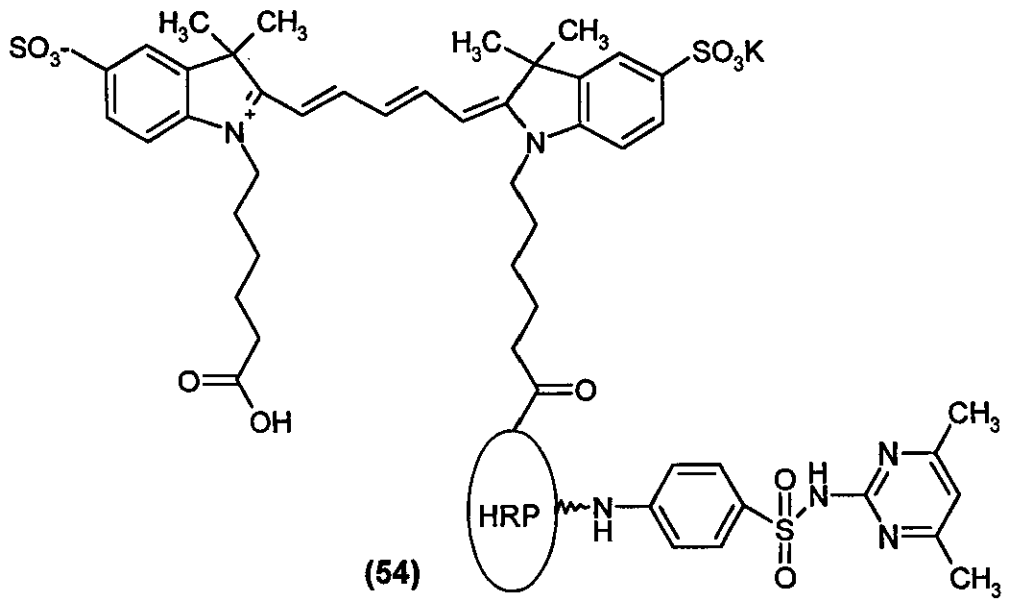
3.4.2 *Preparation of Fluorescein isothiocyanate Labelled Bovine Serum Albumin*[169]



(53)

Bovine serum albumin (20 mg) and *p*-benzoquinone (200 μl, 149 μmol) were dissolved in phosphate buffered saline (PBS, 0.01 M, pH 6), stirred for 2 hours, and dialysed against saline (0.9%, 5 L) for 2.5 hours. The dialysed protein solution was added to a solution of sulfamethazine **(1)** (20 mg, in 1.6 ml PBS : 400 μl DMF) and adjusted to pH 8.0 by the addition of carbonate buffer (0.1 M, pH 9.6). The solution was incubated at 4°C for 36 hours and raised to pH 9.3 with saturated sodium carbonate. Fluorescein isothiocyanate **(34)** (5 mg, 1.26 μmol) was added and the solution incubated for 1 hour, stirring every 10 minutes. Lysine (1 M, 400 μl) was added and the solution stirred for 2 hours. The product was purified using an SG-25 gel column eluting with PBS (0.1 M, 1 ml/min) monitoring at 280 nm. The concentrations of the fluorophore **(34)** and BSA were determined spectrophotometrically and the dye:protein:sulfamethazine ratio calculated as 1:1:2; λ_{max} = 495 nm; ELISA assay $B/B_{0(50\%)} = 7e^{-8}$ M (cf. Sulfamethazine **(1)** = $6e^{-8}$ M)

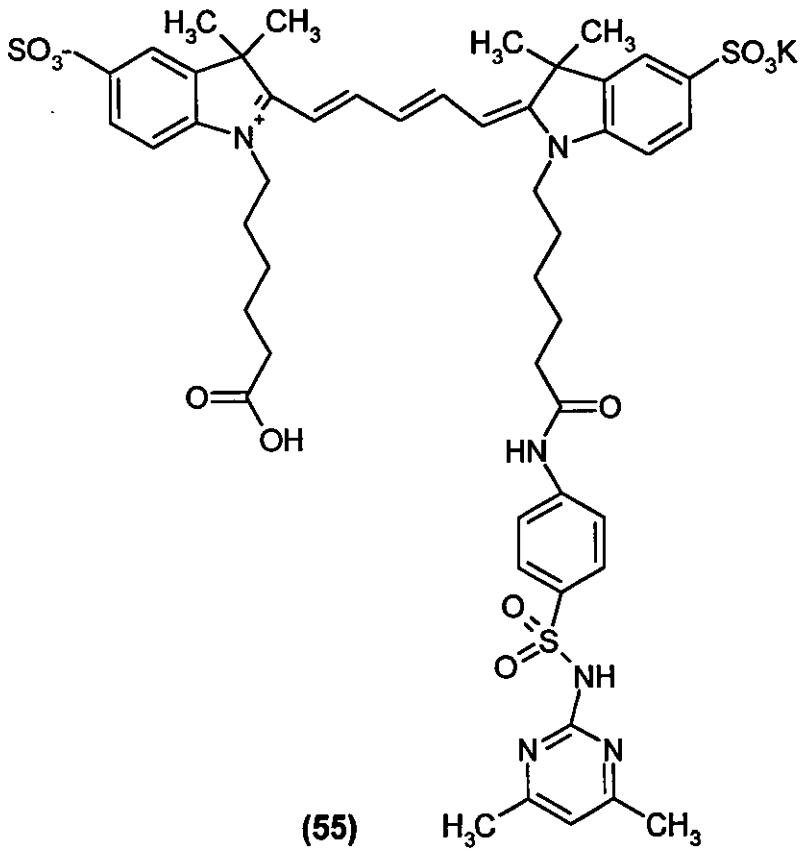
3.4.3 *Preparation of Sulfamethazine-Horseradish Peroxidase-CY5.18.OH*



(54)

A solution of sulfamethazine-horseradish peroxidase **(37A)** (10 ml of 0.95 μmol, pH 5) was added to Cy5.18.OSucc **(47)** (3.7 mg, 3.95 μmol) and stirred for 4 hours at room temperature in the dark. The solution was columned through an SG-50 gel chromatography column (eluting phosphate buffered saline (0.9%, pH 7.2, 5 ml/min) and the product collected. The concentrations of Cy5.18.OH **(35)** and HRP were determined spectrophotometrically and the dye:protein:sulfamethazine ratio calculated as 1:1:2.

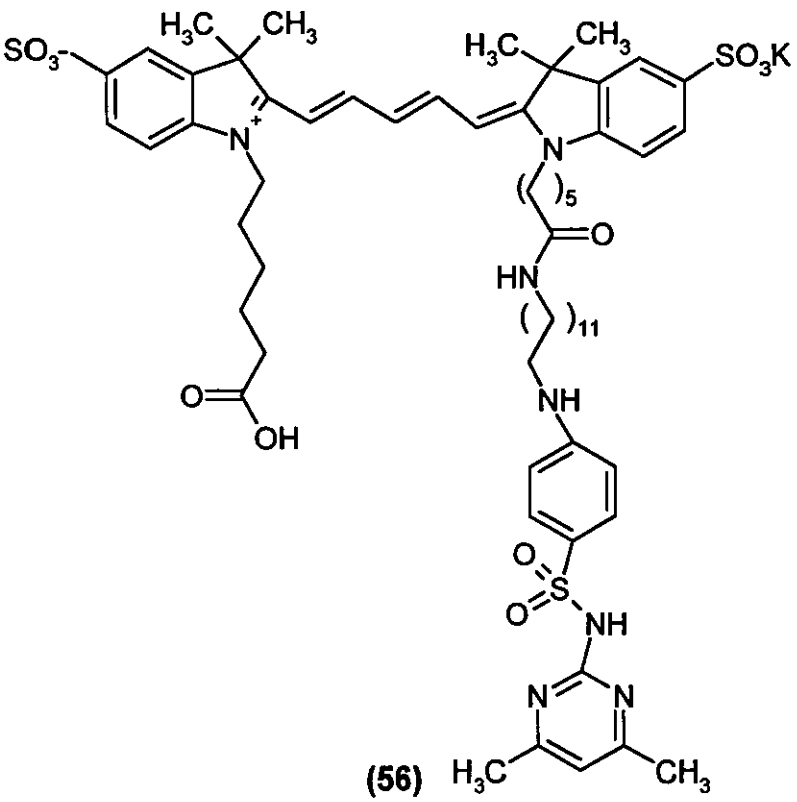
3.4.4 Reaction of Cy5.18.OSucc with Sulfamethazine[165]



(55)

Sulfamethazine **(1)** (10 mg, 35.9 μmol), triethylamine hydrochloride (68 mg, 0.359 mmol) and Cy5.18.OSucc **(47)** (16.8 mg, 17.95 μmol) were dissolved methanol (0.5 ml) and stirred under nitrogen for 16 hours. The solvent was removed and the residue dissolved in methanol (0.1 ml). The product was purified using preparative TLC (79% ethyl acetate/20% methanol/1% acetic acid) and the band at R_f = 0.25 was scrapped off and extracted into methanol. $C_{49}H_{59}O_{11}N_6S_3K$; TLC (80% ethyl acetate/20% methanol): R_f = 0.25; Yield 14%; λ_{max} = 653 nm ε = 44,611 $M^{-1}cm^{-1}$; ELISA assay $B/B_{0(50\%)}$ = $2e^{-7}$ M assuming di-substitution and $1.5e^{-7}$ M for mono-substituted conjugate (cf. sulfamethazine **(1)** = $7e^{-9}$ M).

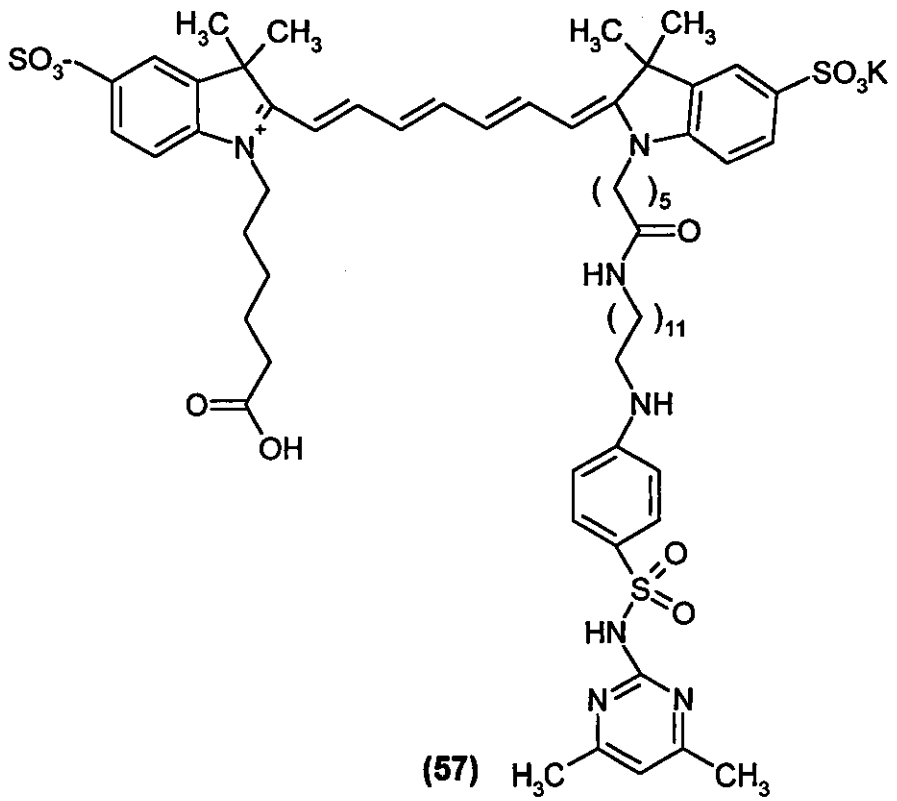
3.4.5 *Reaction of Cy5.18.OSucc with N1-4,6-dimethyl-2-pyrimidinyl-4-[(12-aminododecyl)amino]-1-benzene sulfonamide*



(56)

A solution of Cy5.18.OSucc **(47)** (13 mg, 13.8 μmol), N-hydroxysuccinimide (7 mg) and EDC (15.2 mg) in DMF (0.5 ml) was stirred for 1 hour at room temperature in the dark. N1-4,6-dimethyl-2-pyrimidinyl-4-[(12-aminododecyl)amino]-1-benzene sulfonamide **(38)** (6.5 mg, 14.3 μmol) in DMF (0.5 ml) was added and the solution stirred for a further 2 hours. The solvent was evaporated, the residue dissolved in methanol (0.1 ml) and the solid crystallised from chloroform (2 ml). The blue solid was filtered and dried. $C_{61}H_{84}N_7O_{11}S_3K$ TLC (75% Chloroform/25% methanol): R_f = 0.90; λ_{max} = 650.4 nm; ε = 46,253 $M^{-1}cm^{-1}$; ELISA assay $B/B_{0(50\%)}$ = $2e^{-6}$ M assuming di-substitution $1.65e^{-6}$ M assuming mono-substitution (cf. sulfamethazine **(1)** = $1e^{-8}$ M).

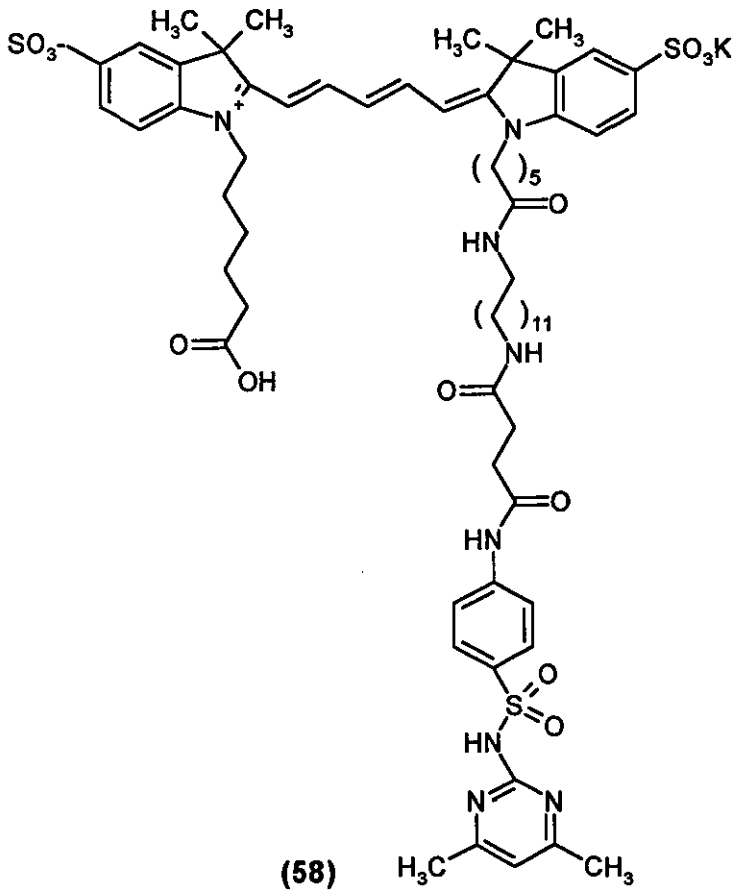
3.4.6 *Reaction of Cy7.18.OSucc with N1-4,6-dimethyl-2-pyrimidinyl-4-[(12-aminododecyl)amino]-1-benzene sulfonamide*



(57)

A solution of Cy7.18.OSucc **(48)** (14 mg, 13.8 μmol), N-hydroxysuccinimide (7 mg) and EDC (15.2 mg) in DMF (0.5 ml) was stirred for 1 hour at room temperature in the dark. N1-4,6-dimethyl-2-pyrimidinyl -4- [(12 - aminododecyl)amino] -1- benzene sulfonamide **(38)** (6.5 mg, 14.3 μmol) in DMF (0.5 ml), was added and the solution stirred for a further 2 hours. The solvent was evaporated, the residue dissolved in methanol (0.1 ml) and the product purified by preparative TLC (37% methanol/63% water) the R_f = 0.95 band was removed and dissolved in methanol. The green solution was filtered. $C_{63}H_{86}N_7O_{11}S_3K$; λ_{max} = 745.4 nm; ELISA assay $B/B_{0(50\%)} = 5e^{-6}$ M assuming di-substitution, $3.6e^{-6}$ M assuming mono-substitution (cf. sulfamethazine **(1)** = $1e^{-8}$ M)

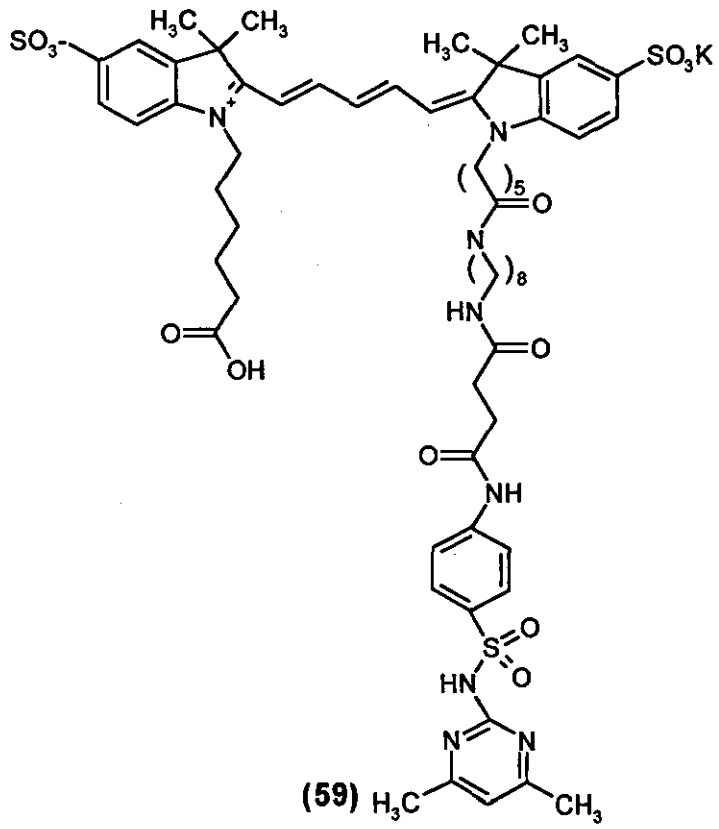
3.4.7 *Reaction of Cy5.18.OSucc with N1-(12-aminododecyl)-N4-(4-{[(4,6-dimethyl-2-pyrimidinyl)amino] sulfonyl}phenyl)succinimide*



(58)

A solution of N1-(12-aminododecyl)-N4-(4-{[(4,6-dimethyl-2-pyrimidinyl)amino]sulfonyl}phenyl)succinimide **(40)** (8 mg, 14.3 μmol), N-hydroxysuccinimide (7 mg) and EDC (15.2 mg) in DMF (0.5 ml) was stirred for 1 hour at room temperature. Cy5.18.OSucc **(47)** (13 mg, 13.8 μmol) was added and the solution stirred for a further 2 hours in the dark. The solvent was evaporated, the residue dissolved in methanol (0.5 ml), and passed through an LH-20 gel filtration column (eluting methanol, 2 ml/min). TLC of band 1 (70% methanol/30% acetic acid) revealed 1 spot, R_f = 0.59. The fractions are pooled and the solvent removed. A blue precipitate formed on addition to phosphate buffered saline (0.9%, pH 7.4). The solid was filtered. The aqueous sample was found to have no sulfamethazine activity (by ELISA) and the remaining fraction was insoluble in aqueous conditions.

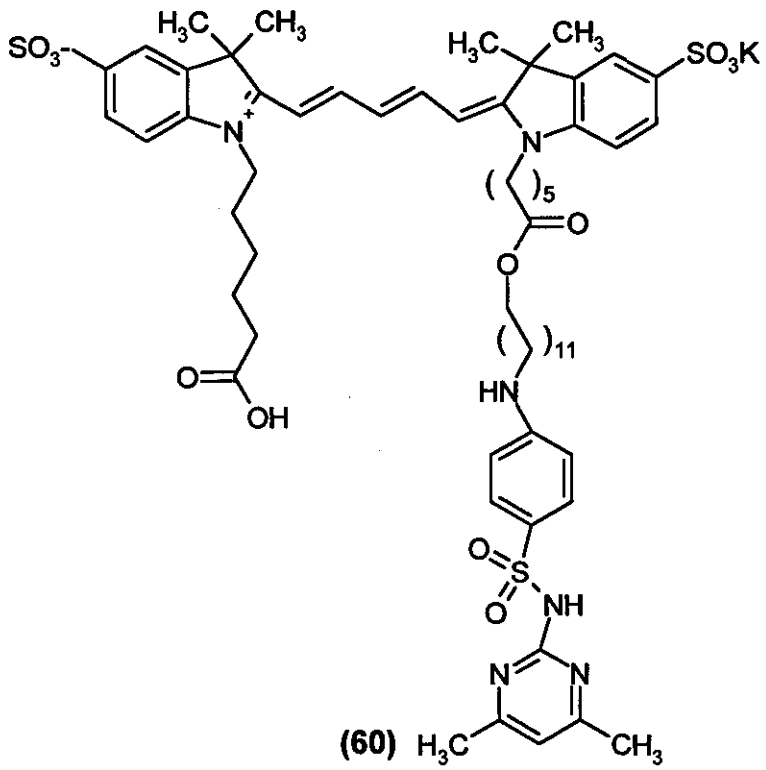
3.4.8 *Reaction with Cy5.18.OH and 4-(4-{[4,6-dimethyl-2-pyrimidinyl)amino]sulfonyl}anilino)-4-oxobutanoic acid using diaminooctane as a spacer*



(59)

A solution of Cy5.18.OH **(35)** (10 mg, 13.5 μmol), 4-(4-{[4,6-dimethyl-2-pyrimidinyl)amino]sulfonyl}anilino)-4-oxobutanoic acid **(39)** (10 mg, 26.9 μmol), EDC (7.7 mg, 40.4 μmol) and N-hydroxysuccinimide (4.5 mg, 40.4 μmol) in dry DMF (20 ml) was stirred in the dark for 2 hours at room temperature. A solution of diaminooctane (4 mg, 27.7 μmol) in DMF (2 ml) was added and the solution stirred in the dark for a further 72 hours. RP_{C-18}TLC (100% methanol) revealed 3 bands. The solvent was removed, the residue was re-dissolved in methanol and passed twice through an LH-20 gel chromatography column (eluting methanol, 2 ml/min then 0.75 ml/min). Further purification by preparative TLC (100% methanol) R_f = 0.77 gave a blue product. $C_{60}H_{79}N_8O_{13}S_3K$; ELISA assay $B/B_{0(50\%)} = 6e^{-6}$ M assuming di-substitution, $4.2e^{-6}$ M assuming mono-substitution (cf. Sulfamethazine **(1)** = $1e^{-8}$ M)

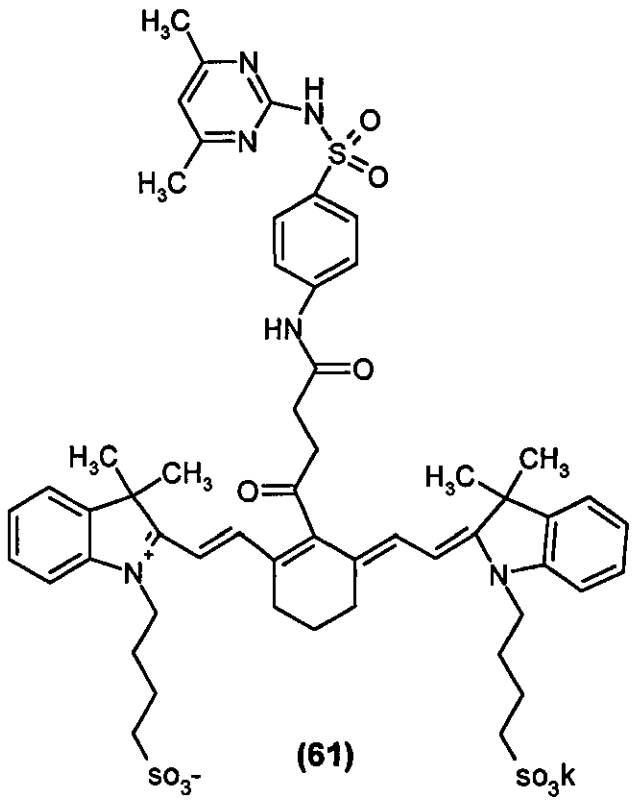
3.4.9 *Reaction with Cy5.18.OH and N1-(4,6-dimethyl-2-pyrimidinyl)-4-[12-bromododecyl)amino]-1-benzene sulfonamide*[165]



(60)

A solution sodium hydroxide (0.2 ml, 25% ($_{w/v}$), 30 mmol) was added to Cy5.18.OH **(35)** (30 mg, 0.04 mmol) in hexamethylphosphorous triamide (HMPT, 2 ml). N1-(4,6-dimethyl-2-pyrimidinyl)-4-[12-bromododecyl) amino]-1-benzene sulfonamide **(42)** (100 mg, 0.16 mmol) was added and the solution stirred for 24 hours. Water (5 ml) was added, the product extracted into diethyl ether (3 x 5 ml), dried ($MgSO_4$) and the solvent removed. The product was purified by preparative TLC (100% methanol) R_f = 0.85 band being removed and extracted into methanol. $C_{61}H_{81}O_{12}N_6S_3K$; λ_{max} = 650.8 nm; ε = 89,315 $M^{-1}cm^{-1}$; ^1H NMR *(400MHz, MeOD-d_4)*: δ 8.54 (s, 3H), 7.87 - 8.33 (m, 12H), 7.42 (s, 2H), 7.34 (d, 4H), 6.7 (t, 1H), 6.36 (d, 4H), 5.9 (s, 2H), 4.57 - 4.84 (m, 3H), 4.05 (d, 2H), 3.48 - 3.66 (m, 4H), 3.3 (s, 6H), 2.6 - 3.1 (m, 4H), 1.75 (s, 12H), 0.84 - 1.90 (m, 22H); ELISA assay: $B/B_{0(50\%)}$ =.1e^{-7} M (cf. Sulfamethazine **(1)** = 6e^{-8} M)

3.4.10 *Attempted Synthesis of 4-(2-(9E)-2-[2-{(E)-1-hydroxymethylidene]-1-cyclohexenyl-4-(4-{[(4,6-dimethyl-2-pyrimidinyl)amino]sulfonyl}anilino)-4-oxobutanoyl]-3,3-dimethyl-2,3-dihydro-1H-2-indolyliden}ethylidene)-1-cyclohexenyl]-1-ethyenyl}-3,3-dimethyl-3H-1-indoliumyl)-1-butane sulfonate*[170]



(61)

A solution of 2-chloro-3-{(E)-1-hydroxymethylidene]-1-cyclohexene-1-carbaldehyde **(49)** (0.378 g, 2.19 mmol) in dry THF was chilled to -78°C. N,N,N',N'-tetramethylethylenediamine (0.36 ml, 2.41 mmol) and n-butyllithium (1.6 M, 1.5 ml, 2.41 mmol) were added and equilibrated for 20 minutes. A chilled solution of succinic anhydride (0.219 g, 2.19 mmol) dissolved in THF (10 ml) was added and the reaction stirred for 2 hours. The reaction was allowed to reach room temperature, quenched with saturated ammonium sulfate solution and extracted into diethyl ether. The ether extract was dried and the solvent removed leaving an orange oily liquid. TLC (100% methanol): R_f = 0.67; Yield 84%.

The product (0.4966 g, 1.955 mmol) was dissolved in DMF (15 ml) and N-hydroxysuccinimide (0.2250 g, 1.955 mmol) with EDC (0.3747 g, 1.955 mmol) were added and stirred for 90 minutes. Sulfamethazine **(1)** (0.5441 g, 1.955 mmol) was added and the solution stirred for a further 2 hours. Water (20 ml) was added, the compound extracted into diethyl ether (5 x 15 ml) and dried. The yellow solid was washed with hot methanol (25 ml), filtered and dried *in-vacuo* leaving an orange oil. TLC (100% methanol): R_f = 0.69; Yield 59%

The product (0.69 g, 1.353 mmol) and 4-(2,3,3-trimethyl-3H-indolio) butanesulfonate **(50)** (0.7984 g, 2.706 mmol) in benzene/butan-1-ol (30 ml : 70 ml) were refluxed using a Dean-Stark condenser at 95°C for 12 hours. The solvents were removed *in vacuo* and the solid was washed with diethyl ether. The solid was dissolved in methanol and passed down an LH-20 gel chromatography column (eluting 75% chloroform/25% methanol, 3.5 ml/min then 1.0 ml/min). The green fragments were collected and the solvents removed *in-vacuo*. $C_{55}H_{63}O_{10}N_6S_3$; TLC (75% chloroform/25% methanol): R_f = 0.23; Yield 75% (Crude), λ_{max} = 782 nm; ε = 61,340 $M^{-1}cm^{-1}$; The ^1H NMR data was not consistent with dye **(51)** or as expected for the required product **(61)**. However, the product was tested by ELISA assay and the results gave significant antibody specificity ($B/B_{0(50\%)}$ = 1.1e^{-7} M (cf. Sulfamethazine **(1)** = 1.0e^{-8} M).

CHAPTER FOUR

IMMUNOASSAY DEVELOPMENT

4.1 Introduction

The development of the immunoassay started with the selection of a solid phase membrane and determination of the blocking and washing conditions. The generation of an immunoassay test strip for the detection of sulfamethazine using a horseradish peroxidase conjugate (HRP) was the first target.

Once a HRP assay was established, the assay conditions were tailored for use with fluorescently labelled sulfamethazine conjugates. Initially a fluorescein conjugate, **(52)**, was used, then pentamethine cyanine dye conjugate (Cy5.18.OH™) **(60)**, and the heptamethine cyanine dye conjugates **(57)** and **(61)** were investigated.

4.1.1 Horseradish Peroxidase Conjugates

Two HRP-conjugates were synthesised. Conjugate **(37A)** was made by a method proposed by Blackmore *et al.*[164] and conjugate **(37B)** by a method developed by Ostermaier *et al.*[29] In the synthesis of conjugate **(37A)**, the carbohydrate groups of HRP were oxidised by sodium periodate to give aldehydes followed by a reductive amination. The product was reacted with diazotised sulfamethazine, and the HRP-conjugate produced was purified by gel filtration chromatography. Using the conjugate in an ELISA assay, as described in Section 2.5.4 checked the activity and antibody specificity. The conjugate was found to be satisfactory although background coloration in the ELISA assay was observed.

In the synthesis of conjugate **(37B)**, the carbohydrate was oxidised to aldehyde groups by *p*-benzoquinone. The product was reacted with sulfamethazine at pH 8.0 and the remaining aldehyde groups were reacted with lysine. The HRP-conjugate produced was purified by dialysis. The activity and antibody specificity were checked by ELISA, and were found to be good, giving a lower background than experienced with conjugate **(37A)**.

4.2 Membrane Sources and Selection

The choice of membranes for an immunoassay depends on several factors: the protein-binding capacity and strength of the polymer, the sensitivity and time required to run the immunoassay, and the type of readout employed. The ability to immobilise proteins is essential, since they are to be used as the immunoassay solid phase, and the strength of the membrane is important for the manufacture and eventual use of a device. The pore size of the membrane is crucial. Polymer membranes with pore sizes from 0.2 to 8 μm are commercially available; the larger the pore size the lower the surface area and therefore the lower protein binding capacity. These large pore membranes yield more rapid assays. Conversely, a smaller pore membrane can bind more antibodies per square centimetre, therefore creating a more sensitive assay. The structure of the membrane is also important as the fluorescence signal from the fluorophore-conjugate is to be read directly off the test strip surface.

Several types of membrane with a range of different pore sizes were obtained from commercial sources (Appendix A.4.1). Their suitability for our purpose was determined by their ability to immobilise antibodies. This was ascertained using the Amido Black protein staining technique.

4.2.1 Amido Black Protein Staining Procedure

Antibody solution (10 μl, 0.45 mg/ml)[§§] was spotted in the centre of the polymer membrane (1 cm^2). The membranes were incubated in amido black staining solution, stirring with an orbital shaker for 10 minutes at room temperature. The membranes were washed twice in de-stain solution (10 ml), with shaking for 15 minutes and the area of stained protein was determined visibly by a panel of three people from different

[§§] In general the concentrations of antibody immobilized on the test strips are expressed as the antibody concentration in mg/ml and the immobilization volume, to avoid the complications of membrane affinity and antibody orientation.

genders and age groups. Particular attention was given to antibody dispersion, even coating, and background clarity. The results were expressed as values of + to +++++ depending on the quality, + indicating poor differentiation between antibody spot and background, +++++ indicated even antibody coating and a clear background. The results are displayed in Appendix A.4.1, and the top five were ranked as shown in Table 4.1.

Table 4.1 Membranes Selected for Use in Further Experiments

Membrane	Pore Size	Manufacturer	Value
Millipore NC – Nitrocellulose	0.45 μm	Millipore	+++++
Immunodyne ABC – Nylon	0.45 μm	Pall	+++++
Biotrace NT – Nitrocellulose	0.45 μm	Gelman	++++
Biodyne-A – Nylon	0.45 μm	Pall Gelman	++++
Fluorotrans – PVDF	0.2 μm	Pall Gelman	+++

The results seem to be highly variable, the variations occurring between membrane types, batches, and manufacturer (Appendix A.4.1). The variations noticed include; irregular antibody dispersion giving an uneven spot, a ring effect where the majority of the antibody resides around the spot, uneven coating, and indiscriminate staining. The staining was distinctly different for nitrocellulose (NC) compared with nylon. Overall, NC gave a clearer background whereas the nylon exhibited some residual staining where no antibody was deposited.

Millipore NC and Immunodyne ABC nylon gave the best overall results, having distinct, round, even coated spots, and a relatively clear background. Biotrace NT (NC), Biodyne-A (nylon) and Fluorotrans (PVDF) membranes gave slight background staining indicated that non-specific interactions of the amido black stain may be occurring. These non-specific interactions can be minimised in the immunoassay by using blocking agents and optimising the test strip washing conditions.

The membranes selected for use in further experiments were given in Table 4.1.

4.2.2 Membrane Liquid Capacity

The liquid capacities for three membranes were determined. A square of each membrane (1 cm^2) was accurately weighed, immersed in water, blotted and re-weighed. The results are displayed in Table 4.2

Table 4.2 Membrane Liquid Capacity

Membrane	Type (Pore size)	Capacity
Millipore NC	Nitrocellulose - 0.45 μm	0.0073 g/cm^2
Pall Immunodyne ABC	Nylon – 0.45 μm	0.0121 g/cm^2
Gelman Biotrace NT	Nitrocellulose - 0.45 μm	0.0088 g/cm^2

It was seen that nylon had a greater liquid capacity than NC, this was consistent with literature findings.[98]

4.3 Membrane Blocking and HRP Conjugate Selection[158]

Blocker is a collective term for various additives that are used prevent non-specific binding but have no active part in the specific immunochemical reaction of the assay. The membranes selected for further evaluation were blocked with polyvinyl alcohol (PVA, 10 K, 1%$_{w/v}$) in phosphate buffered saline (PBS), (0.1 M, 0.9%$_{w/v}$, pH 7.4) or casein (2%$_{w/v}$) in PBS. The ability to prevent non-specific binding by these blocking agents was tested by running an immunoassay with HRP-conjugate **(37B)** and using a TMB chromogenic substrate solution as the mode of detection.

4.3.1 Preparation of Test Strips.

Immunodyne ABC nylon or Millipore NC was stuck onto Autostat heat stabilised polyester cards using double-sided Scotch™ tape and the surface of the membrane was scored into 1 cm^2 squares. An antibody solution (21.5 mg/ml) was diluted by 1/10, 1/50, 1/100, and 1/1000 with PBS to give solutions containing 2.15, 0.43, 0.215, and 0.022 mg/ml

antibody respectively. These solutions (5 μl) were applied to the centre of a test square and the cards dried at 30°C for 30 minutes. One card of each type was blocked by immersion in casein ($2\%_{w/v}$) or PVA ($1\%_{w/v}$) solutions for 30 minutes at 22°C. The cards were dried for 30 minutes at 30°C, cut into strips so as to give the whole antibody concentration range on each strip and stored in dark, dry conditions for up to five days.

4.3.2 Competitive Immunoassay

Sulfamethazine standard (500 μg/l) and PBS control were prepared in semi-skimmed milk. The antibody test strips were submerged in the milk standards (8 ml/strip) for 30 minutes. HRP-conjugate **(37B)** (80 μl, [HRP] = 56 μg/ml) was added to the milk standards and the strips incubated for a further 30 minutes. The strips were washed in Tween-20 solution ($0.25\%_{v/v}$) for 25 minutes with shaking, followed by immersion in a TMB substrate solution. After 2 minutes the reaction was stopped with sulfuric acid solution ($10\%_{v/v}$), and the coloration was determined visibly. Values of – (indicating no colour) to ++++ (intense yellow) were allotted depending on the depth of colour produced. The sulfamethazine positive standard strips and PBS control strips for each blocking condition were compared and the results are given in Table 4.3.

Table 4.3 Determination of Blocking Conditions for Membranes

Test	Type	Blocking	Standard	$^1/_{10}$	$^1/_{50}$	$^1/_{100}$	$^1/_{1000}$
1+	Nylon	Casein	Positive	+	+/-	+/-	-
1	Nylon	Casein	Control	++++	++++	++++	-
2+	Nylon	PVA	Positive	+	-	-	-
2	Nylon	PVA	Control	++++	++++	++++	-
3+	NC	Casein	Positive	-	-	-	-
3	NC	Casein	Control	++	+	+	-
4+	NC	PVA	Positive	-	-	-	-
4	NC	PVA	Control	++++	+++	++	+/-

The assay was competitive as the test strips contained a limited amount of antibody and HRP-conjugate **(37B)**. It was anticipated that the positive strips should have no coloration and the colour generated by the HRP-conjugate on the control strips should decrease with decreasing antibody concentration. The results showed no clear difference between the PVA and casein blocking conditions for the nylon membrane. However, a clear difference between positive and control tests was observed with the PVA blocked NC test strips. The colour intensity on the control test decreased as expected with clear differences seen between each antibody concentration, thus PVA appeared to be the best blocking agent to use with NC. A higher background was seen with HRP-conjugate on the nylon strips compared with the NC, which is in accordance with literature findings and is attributed to non-specific interactions.[98]

4.3.3 Reduction of Immunoassay Incubation Time

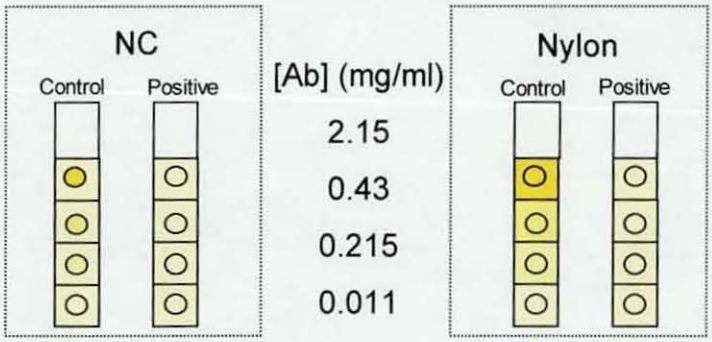
The assay procedure was repeated as described in Section 4.3.2, except to reduce the strip incubation time from 1 hour to 30 minutes the sulfamethazine conjugate was added to the sulfamethazine standards in the milk, before the test strips were incubated. The washing, colouring and stopping procedures were carried out as previously described. There was no visible difference in the results for the two assay procedures, therefore the shorter competitive assay procedure was adopted.

4.3.4 Comparison of Conjugates (37A) and (37B)

A sulfamethazine standard (500 μg/l) and a PBS negative control were prepared in milk. To these standards either conjugate **(37A)** or **(37B)** ([HRP] = 56 μg/ml) was added. The NC and nylon test strips were incubated in the milk standards (8 ml/strip) for 30 minutes, washed in Tween-20 solution (0.25%$_{v/v}$) for 25 minutes with shaking and followed by immersion in a TMB substrate solution for 2 minutes. The reaction was stopped by the addition of sulfuric acid (10%$_{v/v}$), and the coloration was determined visibly.

Conjugate **(37B)** gave the same results as seen previously in Section 4.3.2, Table 4.3. Unfortunately, conjugate **(37A)** exhibited non-specific binding, producing a high background on NC test strips and complete coloration on the nylon strips (Figure 4.1).

Figure 4.1 Illustration of Test Strips Incubated in Conjugate 37A



The non-specific binding of conjugate **(37A)** was due to the interactions of the HRP with the polymer matrix. This was due to the alcohol groups of HRP, produced during the conjugate synthesis, non-specifically binding to the membrane. This non-specific binding was overcome with conjugate **(37B)** because the aldehyde groups of HRP were blocked with lysine during synthesis not reduced as in **(37A)** (Section 4.1). These results indicated that the chosen blocking buffer ($1\%_{w/v}$ PVA) was not compatible with all conjugates, the choice of blocking agent used depends on the properties of the conjugate and have to be optimised for each individual sulfamethazine conjugate. Due to the extensive non-specific binding shown by conjugate **(37A)** it was no longer used.

The remaining HRP experiments were performed to determine if the assay time could be further reduced, to give an estimation of the assay sensitivity, and to give an indication of test strip stability.

4.4 Reduction of the Assay Time[158]

The test strip incubation and washing steps took up the majority of the immunoassay time, so the effects on the HRP-conjugate binding and TMB response on reducing these times were studied.

In the previous experiments the sulfamethazine concentration remained constant and the antibody concentration was varied; to produce a working assay the antibody concentration was fixed and the sulfamethazine concentration varied. An antibody concentration of 0.45 mg/ml was chosen as this gave a strong, but not the maximum, coloration in the previous HRP assay and demonstrated a clear difference between the positive and negative samples.

Immunodyne ABC nylon or Millipore NC were cut into 1 cm x 11.5 cm strips, stuck onto Autostat heat stabilised polyester cards using double-sided Scotch™ tape, and the surface of the membrane scored into 1 cm^2 squares. A solution of antibody (21.5 mg/ml) was diluted (2.22 ml in 50 ml) in PBS giving a concentration of 0.45 mg/ml. This solution (5 µl) was applied to the centre of a test square and the cards dried (30°C, 30 minutes). The cards were blocked by immersion in PVA (1%$_{w/v}$, 22°C, 30 minutes), dried (30°C, 30 minutes) and cut into individual strips, giving one test per strip. These were stored in dark, dry conditions for up to five days.

Sulfamethazine standards 500 µg/l, 100 µg/l and 0 µg/l were prepared in semi-skimmed milk containing the HRP conjugate (80 µl, [HRP] = 56 µg/ml). The nylon and NC test strips were incubated in the milk standards (2 ml/strip) for 5 minutes, washed in Tween-20 solution (0.25%$_{v/v}$) for 10 minute with shaking, followed by immersion in a TMB substrate solution. After 2 minutes the reaction was stopped with sulfuric acid solution (10 µl, 10%$_{v/v}$), and the coloration was determined visibly, allotting values of – (indicating no colour) to ++++ (intense yellow) depending on the depth of colour produced. Control strips were run in parallel having a 30 minute incubation and a 25 minute wash. The results are given in Table 4.4.

Table 4.4 Reduction of Immunoassay Incubation Times

	Millipore NC		Immunodyne ABC Nylon	
	Control	Test	Control	Test
Incubation Time (mins)	30	5	30	5
Wash Time (mins)	25	10	25	10
Substrate Time (mins)	2	2	2	2
Sulfamethazine 500 μg/l	-	-	-	-
Sulfamethazine 100 μg/l	+/-	+/-	+	+
Sulfamethazine 0 μg/l	++++	++++	++++	++++

The test strips gave a coloured response when no sulfamethazine was present, a very pale colour with 100 μg/l and totally clear with 500 μg/l. The change in incubation and washing times gave no visible difference in comparison with the control conditions. Therefore the shorter assay procedure was adopted.

4.5 To Assess the Effect of Sucrose Coating[158]

It was anticipated that coating the test strips with sucrose would extend their shelf-life from 5 days to several months. Therefore the effect of sucrose coating on the immunoassay response was determined.

Immunodyne ABC nylon and Millipore NC assay strips (1 cm^2, 0.45 mg/ml Ab) were prepared as described in Section 4.4. The cards were blocked by immersion in PVA (1%$_{w/v}$, 22°C, 30 minutes) and 50% of the cards were immersed in a sucrose solution (3%$_{w/v}$, 30°C, 30 minutes). The cards were dried for 30 minutes at 30°C, cut into individual strips and stored in desiccated pots (40 strips per pot).

Sulfamethazine standards of 500 μg/l and 0 μg/l were prepared in semi-skimmed milk. The 500 μg/l solution was diluted to give a series of sulfamethazine standards of 250, 125, 62.5, 31.25, 15.5, 8, 4, 2, and 1 μg/l. The HRP conjugate was added to the standard solutions (80 μl, [HRP] = 56 μg/ml) and the test strips incubated (2 ml/strip) for 5 minutes.

The strips were washed in Tween-20 solution (0.25%$_{v/v}$) for 10 minutes with shaking, followed by immersion in a TMB substrate solution. After 2 minutes the reaction was stopped with sulfuric acid solution (100 µl, 10%$_{v/v}$). The colour intensity was determined visibly, assigning values of – (indicating no colour) to ++++ (intense yellow) depending on the depth of colour produced. The results are displayed in Table 4.5.

Table 4.5 Effect of Sucrose Coating

	Millipore NC		Immunodyne ABC Nylon	
Sulfamethazine Concentration	**Control**	**Sucrose**	**Control**	**Sucrose**
500 µg/l	-	-	-	-
250 µg/l	-	-	-	-
125 µg/l	+/-	+/-	+/-	+/-
62.5 µg/l	+/-	+/-	+	+
32.25 µg/l	+	+	+	+
15.5 µg/l	+	+	++	++
8 µg/l	++	++	+++	+++
4 µg/l	++++	++++	++++	++++
2 µg/l	++++	++++	++++	++++
1 µg/l	++++	++++	++++	++++
0 µg/l (blank)	++++	++++	++++	++++

The NC strips gave coloured response when no sulfamethazine was present, getting paler after 8 µg/l, very pale after 62.5 µg/l and totally clear above 250 µg/l, indicating an estimated working region between 60 and 125 µg/l. A similar response was seen for nylon although the change in the colour was more gradual. The addition of sucrose gave no visible difference when compared with the control test, therefore sucrose coating was adopted allowing the test strips to be stored in desiccated pots for up to 3 months.

The NC membrane gave a thin blue line around the antibody spot when the HRP-conjugate was used. This was caused by a higher concentration

of antibody being deposited around the spot. To overcome this problem the method of antibody deposition was changed to total immersion of the membrane in the antibody solution.

4.6 Comparison of ELISA and Test Strip Assay Using Extracts from Spiked Pork

4.6.1 Extraction of Sulfamethazine from Pork.

The MAFF Laboratory in Norwich provided pork samples that had been spiked with sulfamethazine (351 μg/kg). The sulfamethazine was extracted from the pork by two methods, FAPAS Method 18, which is the standard procedure performed at MAFF,[171] or ultrasonication.[33] The sulfamethazine extracts were tested by ELISA assay and antibody coated NC test strips using conjugate **(37B)**.

4.6.1.1 FAPAS Method 18

The porcine muscle sample (10 g) was finely chopped with scissors and placed in a separating funnel. A chloroform : acetone (1:1, 15 ml) mixture was added and the sulfamethazine extracted by shaking. The sample was filtered using Whatman No 1 filter paper and the solids re-extracted with chloroform : acetone (1:1, 15 ml). The filtrates were combined and the solvent removed *in-vacuo*. The residue was ultrasonicated in methanol (1 ml) for 5 minutes and the solution stored in the refrigerator. The solution volume was 0.1 ml per g meat

4.6.1.2 Ultrasonication Method

The porcine muscle sample (10 g) was finely chopped with scissors, divided equally between two containers. PBS (0.1 M, 5 ml) was added to each sample and vortex mixed. The samples were ultrasonicated for 2 minutes followed by centrifugation (5 minutes, 4000 rpm). The

supernatants were removed, re-centrifuged and pooled giving a final volume of 10 ml, which was equivalent to 1 ml per g meat.

4.6.2 Preparation of Test Strips

Millipore NC was cut into 1 cm x 7.5 cm strips, stuck onto Autostat heat stabilised polyester cards using double-sided Scotch™ tape and the surface of the membrane scored into 1 cm^2 squares. The fixed membranes were immersed in an antibody solution (50 ml, 0.45 mg/ml) and incubated at 30°C for 30 minutes. The cards were dried (30°C, 30 minutes), blocked by immersion in PVA (1%$_{w/v}$, 22°C, 30 minutes), sucrose coated (3%$_{w/v}$, 30°C, 30 minutes), dried (30°C, 30 minutes) and cut into individual strips, giving one test per strip. These were stored in desiccated pots for up to 3 months.

4.6.3 Comparison of Extraction Procedures

The muscle extracts were tested initially by ELISA to give an estimation of the sulfamethazine concentration obtained by the standard FAPAS extraction and to compare this with the concentration generated by ultrasonication. The ELISA assay procedure was performed as described in Section 2.5.4 using a sulfamethazine standard range of 100 to 0.19 μg/l ($3.6e^{-7}$ M to $7.03e^{-10}$ M). The FAPAS and ultrasonicated extracts were diluted $^1/_{50}$ and $^1/_5$ respectively, and then further diluted by $^1/_{10}$, $^1/_{50}$, $^1/_{100}$, $^1/_{500}$ and $^1/_{1000}$ to give a series of concentrations. The colour intensities generated in the ELISA by the extracted meat samples were directly compared with those given by the sulfamethazine standards

The intensities generated for the two meat extracts were not significantly different from one another, demonstrating that the extraction methods were comparable. The ELISA results indicated that the diluted extracts contained approximately $2.25e^{-8}$ M sulfamethazine. This corresponded to $1.125e^{-5}$ M sulfamethazine per extract, equivalent to 312 μg/kg meat.

An examination of the extracts using NC test strips was performed as described in Section 4.5 using a sulfamethazine standard range of 500 to 2 μg/l ($1.79e^{-6}$ M to $7.03e^{-9}$ M) and a blank control. The colour intensities generated by the extracted meat samples were directly compared to those given by the sulfamethazine standards.

The sulfamethazine standards generated a range of colour from dark yellow (0 – 2 μg/l), medium yellow (5 – 50 μg/l), light yellow (100 – 200 μg/l) and colourless (> 200 μg/l), having a cut off point at approximately 100 μg/l. We were pleased to find that both of the extracts gave a colourless response thereby suggesting that the sulfamethazine concentrations were above 200 μg/l ($7.19e^{-7}$ M, 200 ppb).

The immunoassay test strips are to be used for sulfamethazine screening and need to detect samples that contain sulfamethazine above the legal limit (100 μg/kg). The encouraging results seen when the HRP-conjugate and the crude test strips produced a positive response made us believe that by optimising the assay conditions a sensitive immunoassay could be achieved.

4.7 Fluoroimmunoassay Development

Although the aim of this research was to develop a competitive fluoroimmunoassay using a solid phase support and a near infrared fluorophore labelled sulfamethazine conjugate, initially the visible fluorophore fluorescein isothiocyanate **(34)** was used as the sulfamethazine label, conjugate **(52)**. This conjugate was used to provide a model system to develop the test procedure and determine the sensitivity of the assay. Patonay *et al.* reported that a solid-phase heterogeneous fluoroimmunoassay would have a good sensitivity.[151]

We have seen previously that when antibodies were immobilised on NC test strips, these could be used to detect sulfamethazine (Section 4.6.3). The tracer used in this assay was the horseradish peroxidase conjugate **(37B)**, which was detected using a TMB chromogenic substrate solution. A sensitive and rapid immunoassay was obtained that used an enzyme system, which had the advantage of detection amplification because the enzymic reaction produced more colour intensity over time. The fluorophore-conjugate system will not have the advantage of signal amplification, and the detection of the fluorescence signal will depend on the fluorophore concentration, possible amplification effects generated by the use of detergents, and will rely on the sensitivity of the instrumentation. Therefore, a fluoroimmunoassay in which the generated fluorescence intensity (I_f) from the labelled antigen decreased with increasing concentration of free antigen was developed.

It was known that the presence of non-specific binding in an immunoassay could have a detrimental effect by decreasing the sensitivity, stability, and reproducibility and increasing the assay response time.[77] To overcome these problems, the blocking reagents, washing conditions and incubation times for the immunoassay were optimised using conjugate **(52)**.

4.7.1 Optical Arrangement

The fluorescence intensity produced by the fluorophore when adsorbed on to the antibody coated test strip was detected using a Spex Fluorolog2 in FF mode and optical fibre holder B (Section 2.6.1). The bifurcated glass fibre used (Oriel, USA) terminated in a randomly arranged fibre bundle that was immobilised in a black box to avoid interference of external light. The fibre bundle had a diameter of 0.4 cm^2, which gave an illumination area of 0.125 cm^2 on the membrane strip when placed in the strip holder at 90° to the fibre end (Figure 2.6). The membranes had capacities of 0.0088, 0.0073 and 0.0121 ml/cm^2 for Millipore NC, Biotrace NT and Immunodyne ABC nylon; respectively (Section 4.3.2) and the antibody concentrations investigated ranged from 0.0125 mg/ml to 5 mg/ml.

The theoretical concentrations of fluorophore that could be determined on these solid-phase matrices ranged from $5.04e^{-8}$ M to $9.17e^{-11}$ M and the highest fluorophore concentration was calculated for the nylon strips. This may prove to be advantageous for the assay sensitivity, as the intensity of fluorescence will be a function of the fluorophore concentration.[172] It has been reported that conventional fluorescent-label immunoassay technologies are limited to detection limits in the $1e^{-9}$ to $1e^{-10}$ mol/l range because of high background readings associated with light scattering and fluorescence by the sample reagents and the solid phase.[175]

When using near infrared fluorophores in a laser diode system with the light source and detector matched to the sample excitation and emission parameters, we have seen detection limits of $1e^{-15}$ mol/l in solution. Although in solid phase fluorimetry the illumination and detection systems have a different geometry than the solution arrangement, this is not necessarily less efficient.[173] The light source and detector can in effect be very close to the sample and thus have good light gathering properties and reduced light scattering; and these fluorescent tracers have the potential to provide many photons of emitted light per molecule, because the cycle of excitation and fluorescence can be repeated many times for a single molecule during the measurement period.

4.7.2 Light Scattering and Autofluorescence of Membranes

There are some major disadvantages associated with using solid-phase immunoassays, namely, light scattering from the membrane material and difficulties in immobilising the antibody uniformly and consistently between batches;[174] both of these effects will generate a higher background fluorescence.

The light scattering and matrix autofluorescence of Millipore NC, Biotrace NT and Immunodyne ABC nylon membranes were tested. This was to determine if significant light scattering occurred on excitation and to check if inherent membrane fluorescence was present, which would interfere with the fluorescence spectrum generated by the fluorophore.

The polymer membranes were prepared for testing by mounting onto heat stabilised polyester cards using double-sided Scotch™ tape and then immersion in PBS for 30 minutes. The test strips were blocked, sucrose coated and dried as described in Section 4.6.2. The background emission spectra were generated using the following parameters displayed in Table 4.6 and the results are given in Table 4.7.

Table 4.6 Fluorimeter Parameters Used to Determine Light Scattering and Matrix Autofluorescence

Excitation	483, 493, 503 nm	**Emission**	500 - 600 nm	**Increment**	1 nm
Integration	1 s	**Slits – Excitation**	2.5, 2.5 mm (9.0, 9.0 nm)		
		Emission	1.25, 1.25 mm (4.5, 4.5 nm)		

Table 4.7 Fluorescence Intensities Generated by Polymer Matrices

Membrane Type	Background Intensity at 525 nm
Millipore NC	11910 cps
Biotrace NT	28350 cps
Immunodyne ABC 0.45 μm pore	13480 cps

Similarly the emission spectra of antibody coated membranes excited at 493 nm were obtained, which gave the background fluorescence intensities generated by the immobilised Ab. The strips were prepared as described in Section 4.6.2 and the spectra acquired using the conditions outlined in Table 4.6. The results are given in Table 4.8 and the spectra are displayed in Figure 4.2 as a plot of Fluorescence Intensity (cps) Vs Wavelength (nm)

Table 4. 8 Fluorescence Intensities Generated upon Excitation of 0.1 mg/ml Antibody Immobilised on Polymer Matrices

	Intensity at 530 nm	Intensity at 550 nm
*Millipore NC	$6.00e^3$ cps	-
Immunodyne ABC 1.2 μm pore	$5.10e^6$ cps	$4.15e^6$ cps
Immunodyne ABC 0.45 μm pore	$4.20e^6$ cps	$4.35e^6$ cps
Fluorotrans	-	$4.73e^6$ cps
Biodyne-A	$5.20e^6$ cps	$4.15e^6$ cps

Figure 4.2 Emission Spectra of 0.1 mg/ml Antibody Coated Polymer Membrane Test Strips

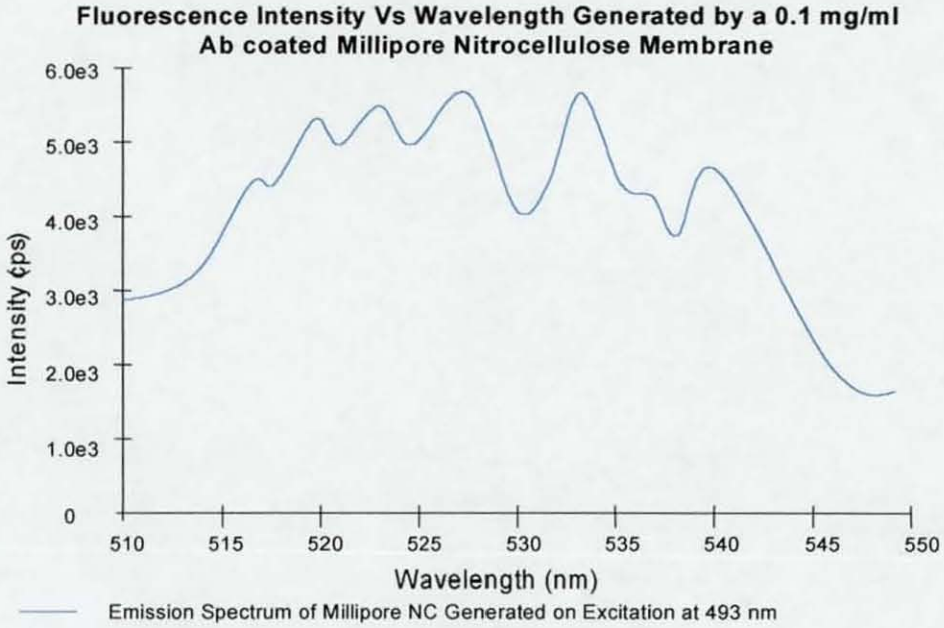
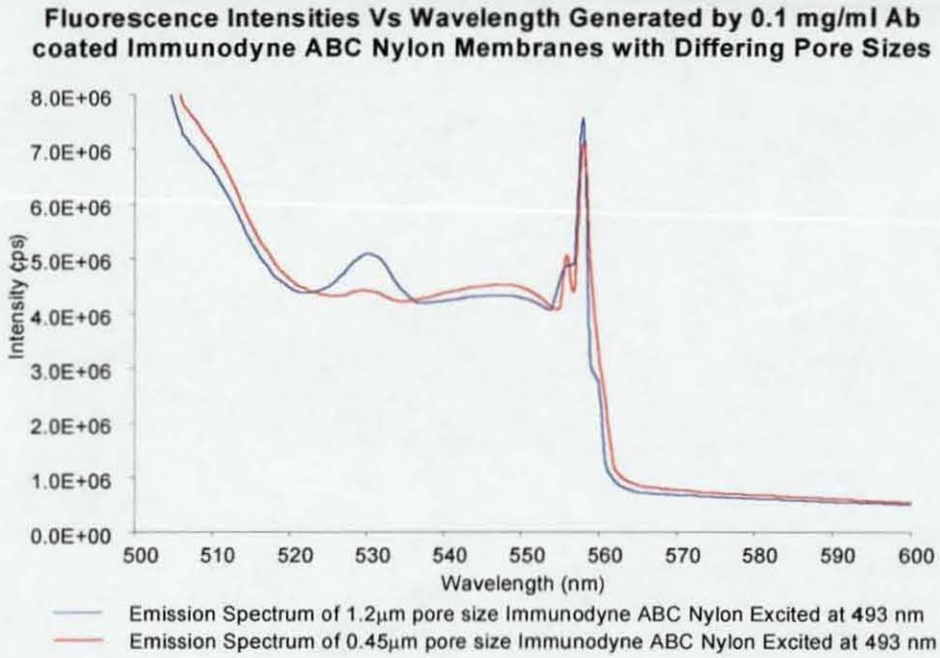
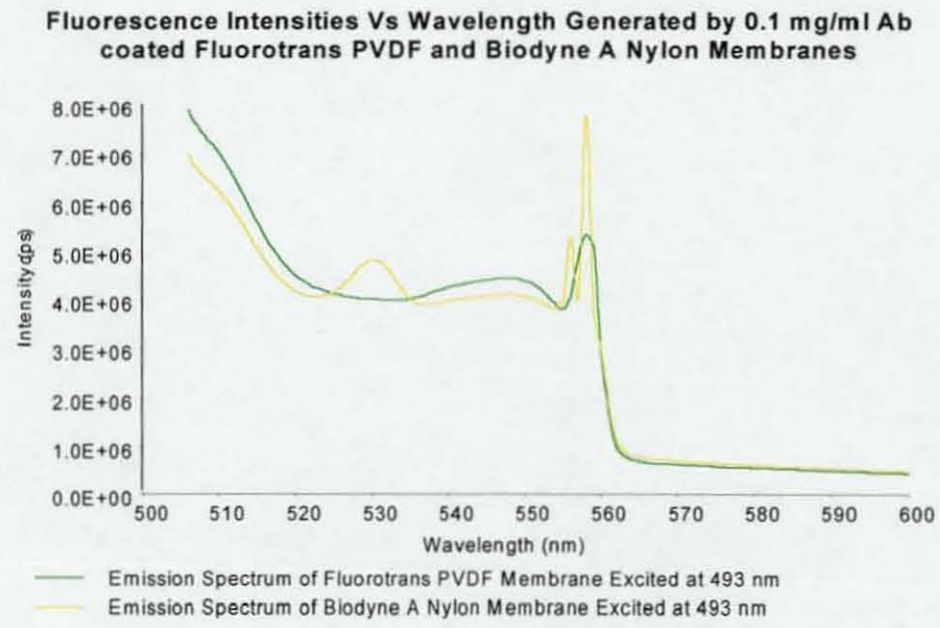


Figure 4.2 Emission Spectra of 0.1 mg/ml Antibody Coated Polymer Membrane Test Strips





The Millipore NC, Biotrace NT and the Immunodyne ABC nylon membranes (0.45 μm pore) showed no autofluorescence when excited at 493 nm and very little Raman and Rayleigh scattering due to the PBS solution, background intensities in the range of $1e^4$ – $3e^4$ cps were generated.

When the strips were coated in 0.1 mg/ml Ab, emission peaks were seen at 530 and 550 nm. The peak at 530 nm was due to the antibodies as they exhibit an emission spectrum between 280 and 600 nm adding to the background.[175] The peak at 550 nm was probably due to Rayleigh light scattering from the residual solvent molecules present on the membrane. Patonay *et al.* used NC in their solid-phase fluoroimmunoassay research because of its high protein binding affinity and low reflective surface.[105] We also saw a low reflection on excitation of Millipore NC, which generated a peak at 525 nm with an intensity of $6e^3$ (cps); unfortunately this spectrum was obtained using smaller slits around the excitation monochromator, (0.5, 0.5, 1.25, 1.25,), and therefore cannot be directly compared with the other spectra.

The excitation of Immunodyne ABC (1.2 μm pore) and Biodyne-A nylon membranes gave peaks at 530 nm with intensities of $5.1e^6$ cps; we decided that such a high background autofluorescence would interfere with the fluorescence intensity generated by the fluorophore so the use of these membranes was not pursued. The manufacturers claimed that Fluorotrans™ offers a lower background than Biodyne-A and Immunodyne ABC, this was confirmed, as the membrane did not give rise to a peak at 530 nm. Therefore Millipore NC, Fluorotrans PVDF and (0.45 μm pore) Immunodyne ABC nylon membranes were used in later experiments.

4.7.3 Fluorescein Dye Conjugates

4.7.3.1 Sulfamethazine Labelling

Both direct and indirect labelled sulfamethazine conjugates were synthesised using fluorescein isothiocyanate **(34)**. This visible fluorophore was chosen because it was commercially available and contains an

isothiocyanate group that can form a conjugate with sulfamethazine. The directly labelled conjugate **(52)** was made by the reaction of the isothiocyanate group with the primary amine group of the sulfamethazine to give a thiourea in 42% yield. The molar absorptivity of this product was reduced to 32,086 $M^{-1}cm^{-1}$ from 67,000 $M^{-1}cm^{-1}$ of the parent dye, and although the expected reduction in antibody specificity was observed, the conjugate **(52)** was only 1.6 times less active than sulfamethazine. This was an encouraging result and the conjugate was used in the development of a competitive immunoassay for sulfamethazine detection. The indirectly labelled sulfamethazine **(53)** was made by reaction of activated bovine serum albumin (BSA) with sulfamethazine followed by the reaction of this product with fluorescein isothiocyanate **(34)** at pH 9.0. Finally the product was mixed with lysine and the resulting conjugate separated by gel filtration chromatography. Conjugate **(53)** produced had a dye: protein: sulfamethazine ratio of 1: 1: 2 and an antibody specificity of 1.2 times less active than sulfamethazine. This conjugate **(53)** was also suitable for use in further immunoassay development, however, Patonay *et al.* proposed that the directly linked fluorophore would produce a smaller molecular size conjugate; which would be advantageous in an immunoassay because the smaller sized label produced would not sterically interfere with the antigen-antibody binding.[105] Bhattacharyya suggested that direct linkage of an antigen with fluorescein isothiocyanate **(34)** would give a faster conjugation reaction than with indirect labelling using proteins.[130] Therefore the directly linked fluorescein-conjugate **(52)** was used throughout the fluoroimmunoassay development

4.7.3.2 Fluorescence Spectra of Conjugate (52)

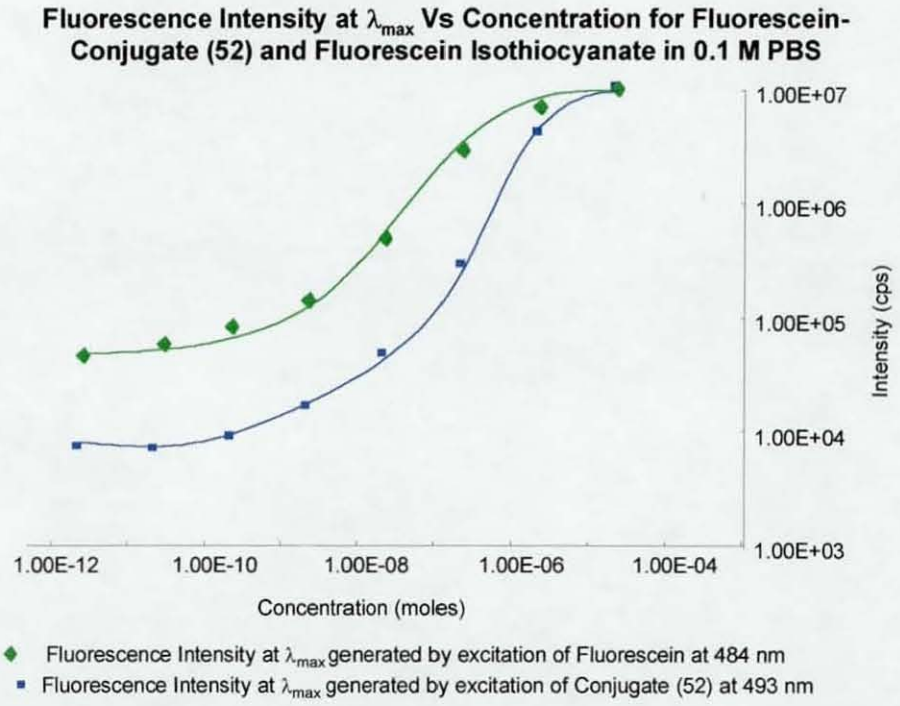
The fluorescence intensity generated from the fluorophores in solution were detected using the Spex Fluorolog2 in RA mode, with cuvette holder A (Section 2.6.1) and disposable 5 ml plastic cuvettes (1 cm x 1 cm x 5 cm). A series of solutions containing conjugate **(52)** or fluorescein isothiocyanate **(34)** were made in PBS (0.1 M, pH 7.4) and the

fluorescence intensities of these solutions were determined by running an emission scan with the following parameters (Table 4.9). The results are displayed in Figure 4.3 as a plot of the maximum Fluorescence Intensity (cps) Vs Fluorophore Concentration (moles).

Table 4.9 Parameters Used to Generate Solution Emission Spectra

Excitation Wavelengths		**Emission Wavelength**			400 – 600 nm
Fluorescein **(34)**	484.1 nm	**Increment**	1 nm	**Integration**	0.5 s
Conjugate **(52)**	492.7 nm	**Slits**		1.25 mm all round	

Figure 4.3 Comparison of the Fluorescence Intensities Generated by Fluorescein Isothiocyanate (34) and Conjugate (52) in 0.1M PBS Solution

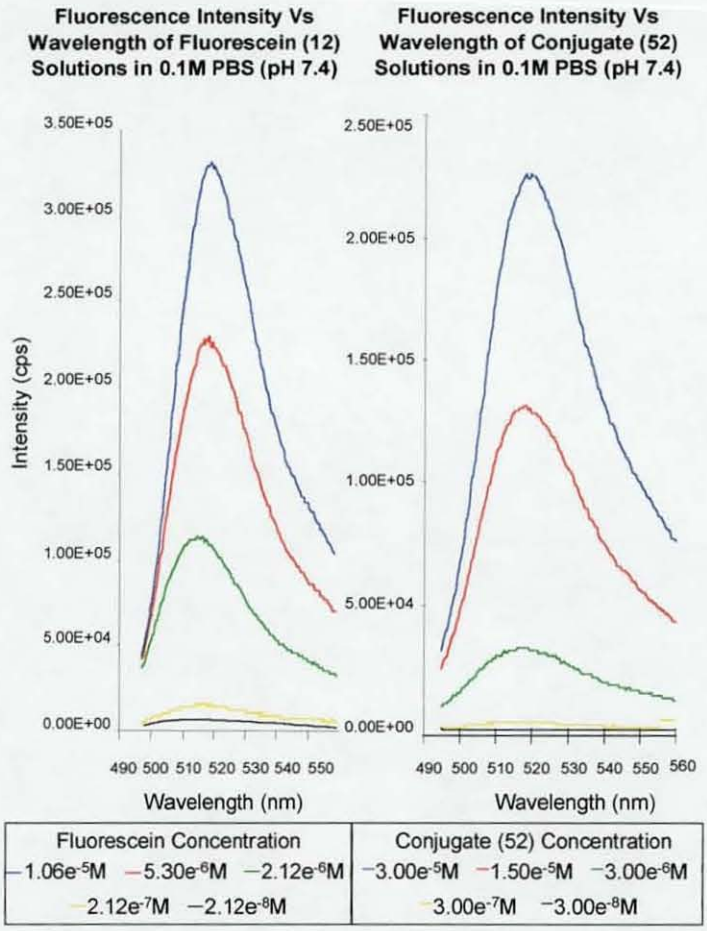


The fluorescein isothiocyanate **(34)** was excited at 484.1 nm and had an emission maximum at 520 nm, whereas the fluorescein conjugate **(52)** was excited at 492.7 nm and gave the same emission maximum, the conjugation of sulfamethazine to fluorescein isothiocyanate **(34)** has reduced the Stokes' shift by 8.6 nm. It is seen from the Figure 4.3 that the fluorescence intensities (I_f) generated by the same concentrations of fluorescein **(34)** and the conjugate **(52)** differ greatly due to the reduced

molar absorptivity and a possible reduction in the quantum efficiency when fluorescein isothiocyanate **(34)** is conjugated to sulfamethazine.

On comparing the fluorescence spectra of fluorescein **(12)** and the fluorescein-conjugate **(52)** it was possible to estimate the relative quantum efficiency of the conjugate (Figure 4.4).

Figure 4.4 Comparison of the Emission Spectra of Fluorescein (12) and Conjugate (52) in 0.1 M PBS Solution



Absolute determinations of the quantum efficiency are difficult to perform. It is essential to use corrected spectra, to ensure the absence of inner filter and energy transfer effects, and to take into account the fluorescence polarisation phenomena. Therefore an approximate Φ is calculated by comparing the emission spectra of fluorescein **(12)** and conjugate **(52)** (Figure 4.4) and using Equation 1.3; $I_f = 2.3\Phi I_0 \varepsilon bc$.

Given that I_0 and b were equal for both fluorophores in the experiment, the fluorescein fluorophore **(12)** gave $I_f = 2.29e^5$ cps at concentration of

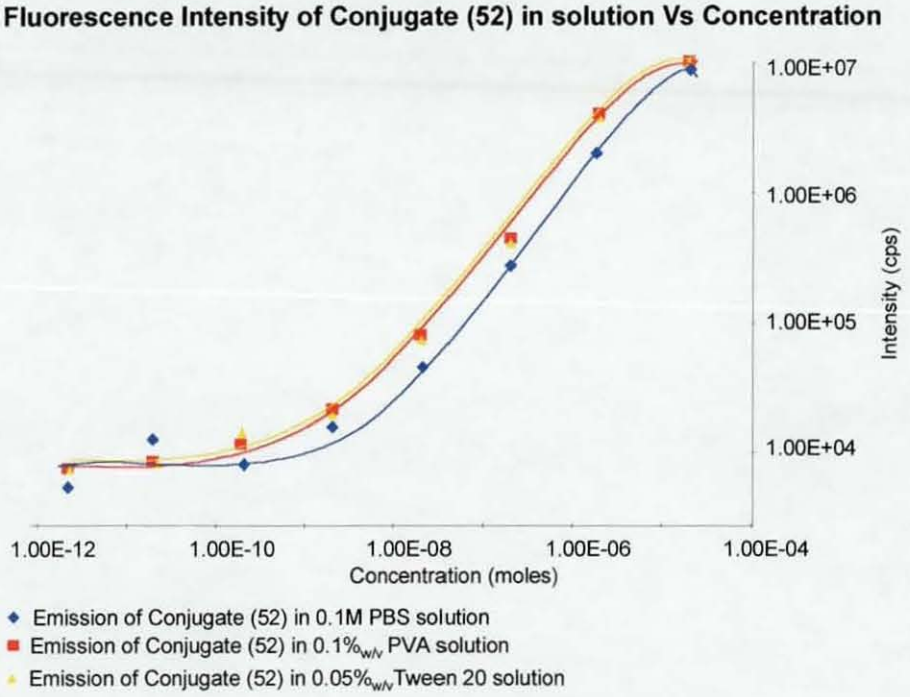
$5.3e^{-6}$ M, with $\Phi = 0.85$, $\varepsilon = 67{,}000\ M^{-1}cm^{-1}$.[175] $3.0e^{-6}$ M fluorescein-sulfamethazine **(52)** gave an intensity of $I_f = 3.25e^{4}$ cps and $\varepsilon = 31000\ M^{-1}cm^{-1}$, therefore Φ was calculated as 0.51.

It had been expected that on conjugation to sulfamethazine the molar absorptivity and quantum efficiency of the fluorophore would decrease and give a compound that was not as efficient as the original fluorophore. Several studies on the luminescence of dyes on solid surfaces have been reported but there is no detailed account on the dependence of intensity of emission on the amount of radiation absorbed by the dye. Quantitative determinations of fluorescence quantum efficiencies in these kinds of systems are therefore not found in the literature.[178,176]

Patonay *et al* have proposed that detecting the fluorescence intensity of the conjugate on a solid phase matrix should allow the generation of a stronger detection signal than experienced in solution, due to the concentrating effect on the fluorophore in the detection area and a reduction of the quenching effects of solvent molecules.[151] The fluorescence intensity could also be improved by the addition of certain detergents, for example Triton x-100. This is known to form micelles that encapsulate the fluorophore, protecting it from collisional quenching, which results in an enhancement of the fluorescence intensity.[133]

The effect on the fluorescence intensity of the fluorescein-conjugate **(52)** in solution on addition of PVA ($0.1\%_{w/v}$ 10K) or Tween-20 ($0.05\%_{w/v}$) were investigated. The emission spectra at different conjugate concentrations were obtained using the method described above and the results are displayed in Figure 4.5 as a plot of the maximum Intensity (cps) Vs Concentration (moles).

Figure 4.5 Comparison of the Fluorescence Intensities Generated by Conjugate (52) in 0.1 M PBS, 0.1%$_{w/v}$ PVA (10K) and 0.05%$_{w/v}$ Tween-20 Solutions



The fluorescence intensity of conjugate **(52)** was enhanced by 76% when dissolved in PVA and by 100% when dissolved in Tween-20 solution. The increase in fluorescence intensity observed in the PVA solution was due to a change in solvent viscosity, which reduces the collisional quenching effects of the conjugate. Similarly, the enhanced fluorescence seen with the addition of Tween-20 was due to the reduction in collisional quenching by the formation of micelles that encapsulated the conjugate.[177] As the PVA or Tween-20 did not detrimentally affect the fluorescence intensity of the conjugate, these blocking and washing reagents were suitable for use in the initial development of the test strip immunoassay.

4.7.4 Fluorescence of Fluorescein Conjugate (52) on NC Test Strips

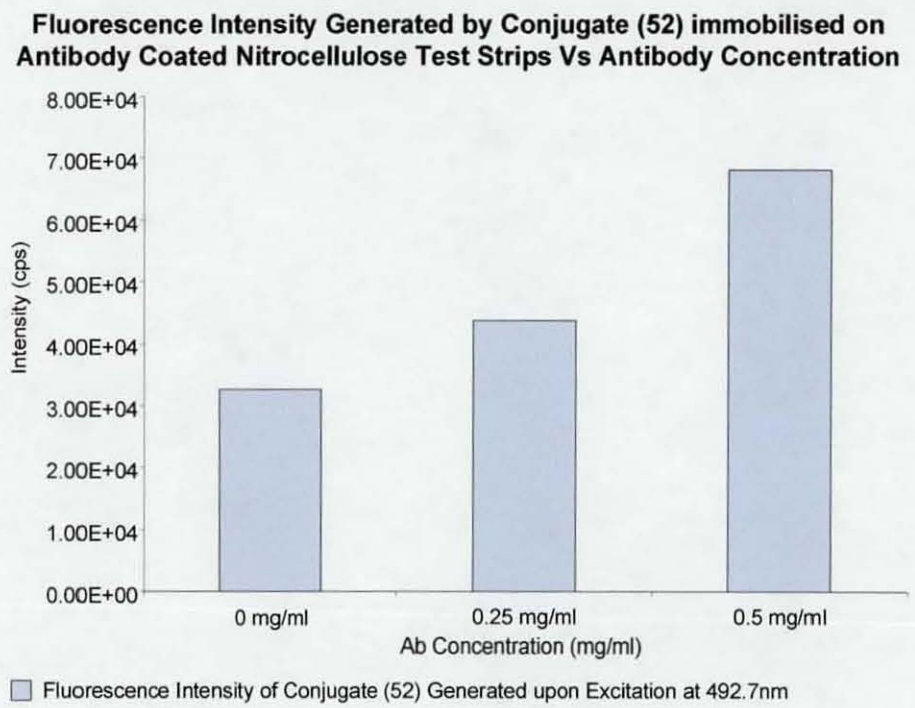
In all solid-phase experiments an antibody coated strip, incubated in PBS, was used to give a blank background spectrum. This control test was performed with each set of experiments and the fluorescence intensity generated by the fluorophore was divided by the blank spectrum to give the F/B ratio.

The fluorescence intensity generated by the conjugate **(52)** when immobilised on an antibody coated NC test strip was studied. Millipore NC test strips with antibody concentrations of 0, 0.25 and 0.5 mg/ml were prepared as described in Section 4.6.2. These test strips were incubated in the conjugate solution (2 ml, $7.987e^{-5}$ M) for 30 minutes, washed in Tween-20 (2 ml, $0.05\%_{w/v}$) for 10 minutes by orbital shaking and blotted on a tissue. The intensity was determined by an emission scan using the following parameters (Table 4.10) and the results are displayed in Figure 4.6 as a plot of maximum Intensity (cps) Vs Antibody Concentration (mg/ml).

Table 4.10 Parameters Used to Generate the Fluorescence Intensities of Conjugate (52) on a Solid Surface

Excitation	492.7 nm	**Emission**	500 – 600 nm	**Increment**	1 nm
Integration	0.5 s	**Slits – Excitation**	1.25, 0.5 mm (4.5, 1.8 nm)		
		Emission	0.5, 1.25 mm (1.8, 4.5 nm)		

Figure 4.6 Comparison of the Fluorescence Intensities Produced by Conjugate (52) Immobilised on Nitrocellulose Test Strips



It was found that the fluorescence intensity increased with increasing antibody concentration. However, the intensity originating from the conjugate on the 0 mg/ml antibody coated test strip, was due solely to non-specific binding to the membrane. It appeared that the fluorescence intensities generated on the antibody coated strips were a combination of non-specific and specific antibody binding. As indicated previously (Section 4.3), the non-specific binding would be reduced by tailoring the blocking and washing conditions with the conjugate used.

4.7.5 Optimisation of Fluorimeter Slits and Angle of Detection

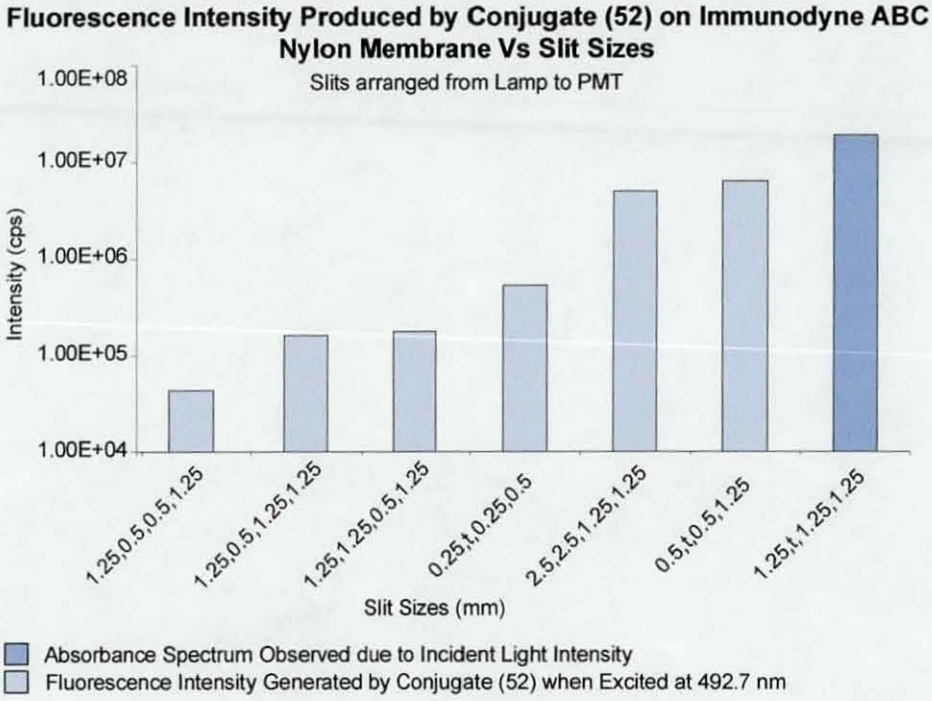
Before the solid phase immunoassay conditions were optimised, the instrumentation parameters and solid phase illumination geometry were investigated. To determine the ideal fluorimeter parameters, the relationship between the conjugate fluorescence and incident light intensities were examined.

Immunodyne ABC nylon test strips (0.5 mg/ml Ab) were prepared as outlined in Section 4.6.2 and incubated in fluorescein-conjugate **(52)** (2 ml, $7.987e^{-5}$ M) as described in Section 4.7.4. An emission scan using the following parameters (Table 4.11) was performed and the results are displayed in Figure 4.7 as a plot of Fluorescence Intensity (cps) Vs Slit Sizes (mm).

Table 4. 11 Conditions Used to Determine the Optimal Fluorimeter Parameters for Solid Phase Emission Spectra

Excitation	492.7 nm	**Emission**	500 – 600 nm	**Increment**	1 nm
Integration	0.5 s	**Slits – Excitation**	Varied from 0.25 – 2.5		
		Emission	Included t (target slit)		

Figure 4.7 Relationship Between the Fluorescence Intensity of Conjugate (52) and Fluorimeter Slit Sizes

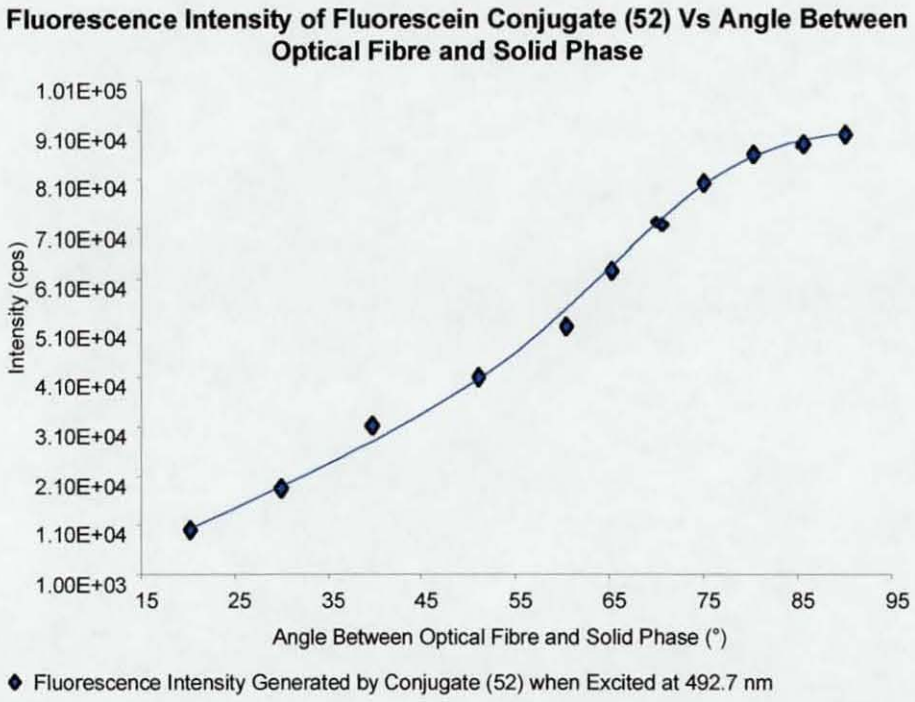


As expected the fluorescence intensity increased with increasing slit size, as the slit sizes determine the bandpass and, when coupled with the scanning integration time, controls the intensity of the incident light that reached the solid surface. Changing the slits around the excitation monochromator exhibited the greatest effect on the fluorescence intensity, this was seen when comparing 1.25, 0.5, 1.25, 1.25 with 1.25, 1.25, 0.5, 1.25 slit arrangements, the latter gave the greater fluorescence intensity (8.81% increase). When a target slit (t) was introduced into the post excitation monochromator slot the intensity was greatly improved. This allowed the first slit size to be decreased to give a smaller bandpass but gave no control of the bandpass before the sample. When the 1.25, t, 1.25, 1.25 slit arrangement was tried the fluorophore gave an absorbance spectrum, due to the light being too intense, and caused the sample to be photo-bleached. The slit arrangements chosen for further use were 2.5, 2.5, 1.25, 1.25 and 0.5, t, 0.5, 1.25 as these gave sufficiently high fluorescence without photobleaching the conjugate.

In order to minimise the detrimental effects of reflected and scattered light, the relationships between the angle of the optical fibre light source to the solid membrane surface were investigated.

The test strips were incubated in fluorescein-conjugate **(52)** as described above and were fixed into the sample holder at varying angles to the optical fibre (Section 2.6.1, Figure 2.6). The maximum fluorescence intensity generated by conjugate was acquired by an emission spectrum using the parameters outlined in Table 4.11 (slits set at 2.5, 2.5, 1.25, 1.25) and the findings of this experiment are shown in Figure 4.8 as a plot of Intensity (cps) Vs Angle to the Detector(°).

Figure 4.8 Relationships Between the Angle of the Optical Fibre Light Source to the Solid Surface



As the angle to the optical fibre decreased the fluorescence intensity decreased. The intensity was fairly constant when angles between 80° and 90° were used, however a sharp decrease occurred when angles less than 80° were used. This may be due to the distance changing between the test strip and the fibre tip. Placing the membrane at 90° to the fibre tip does not seem to enhance the problem of scattered or reflected light, which was attributed to the random arrangement of the optical fibres that

allowed the fluorescence signal to be indirectly transmitted to the PMT. This optical arrangement was used hereafter.

Patonay *et al.* found that the largest F/B ratio for their near infrared fluorophore **(51)** was observed when detected at 64° to the light source.[151] The angle that they determined was measured between a light emitting laser diode and a photodiode detector that were housed together, therefore our experiments are not directly comparable. It was anticipated that the angle of detection would become more important when near infrared dyes are used, as the fluorescent lifetimes of the dyes are longer than that of membrane and the membranes themselves can be photo-bleached;[105] thus making fluorescence intensities of the near infrared conjugates more instrument dependent.

4.7.6 Fluorescence Generated on NC and Nylon Test Strips

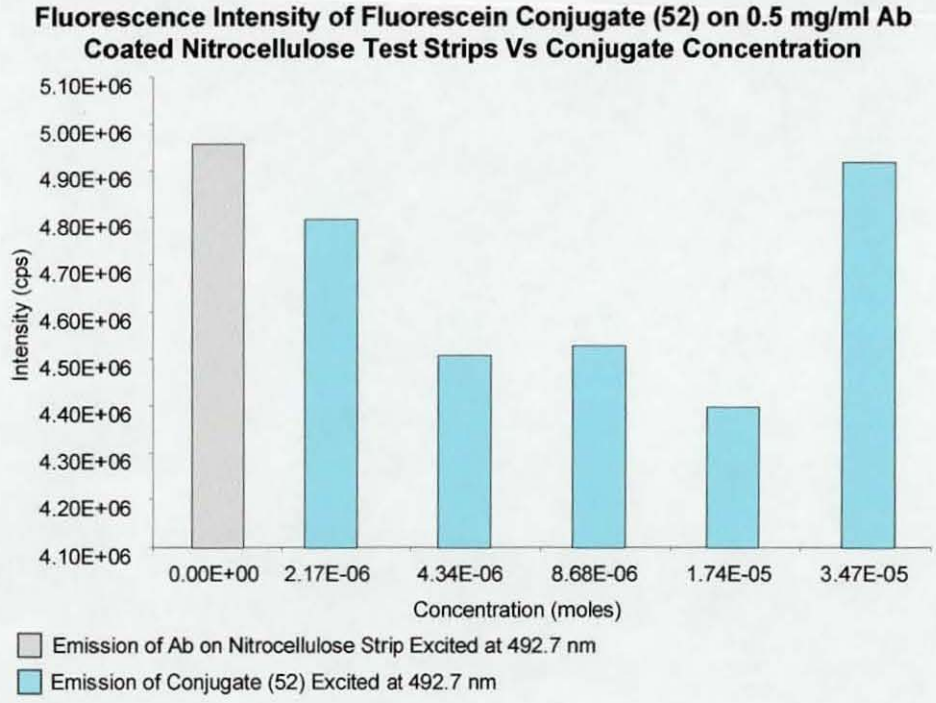
The fluorescence intensities generated by a series of fluorescein-conjugate **(52)** concentrations were used to produce a concentration curve. This was intended to verify that the slits and angle were appropriate for the assay, and to give an indication of a suitable conjugate concentration for use in the immunoassay.

Millipore NC, Immunodyne ABC nylon and Fluorotrans PVDF test strips (0.5 mg/ml Ab) were prepared as outlined in Section 4.6.2. These strips were incubated for 5 minutes in a conjugate **(52)** solution (2 ml, $3.47e^{-5}$ M to $2.17e^{-6}$ M) or a PBS blank, washed in Tween-20 (2 ml) for 10 minutes with orbital shaking and blotted on a tissue. The fluorescence intensity was determined by an emission scan using the following parameters (Table 4.12), the complete results are given in Table 4.13; the concentration curves generated on the NC and Nylon test strips are given in Figures 4.9 and 4.10 and the Fluorotrans results are displayed in Figure 4.11.

Table 4. 12 Fluorimeter Parameters Used to Generate an Emission Spectrum of Conjugate (52) on a Solid Surface

Excitation	492.7 nm	**Emission**	500 – 600 nm	**Increment**	1 nm
Integration	0.5 s	**Slits – Excitation**		0.5, t,	
		Emission		0.5, 1.25	

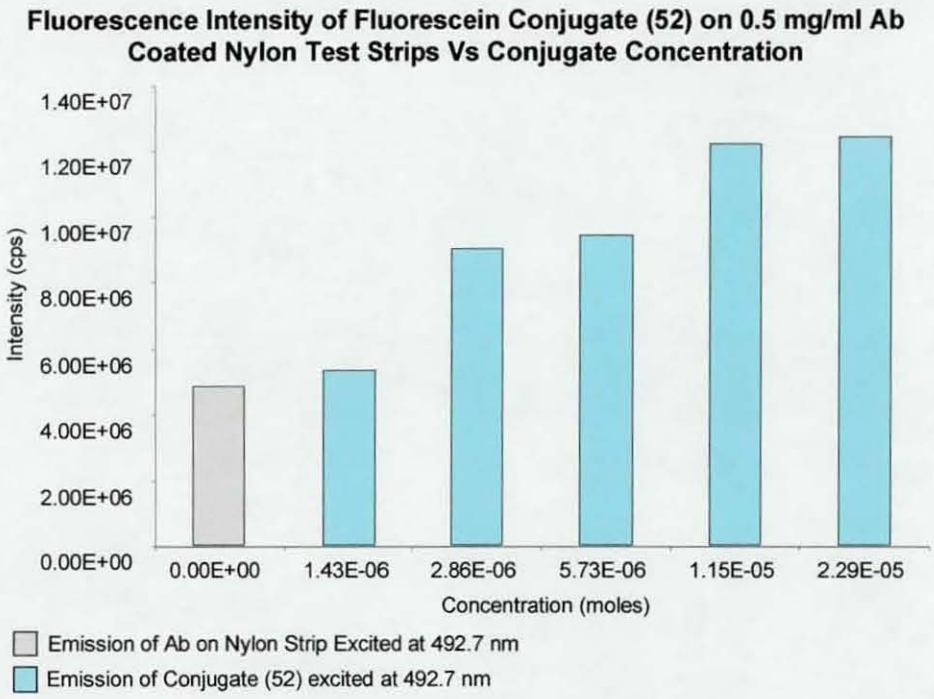
Figure 4.9 Concentration Curve Generated by Conjugate (52) on 0.05 mg/ml Antibody Coated Millipore NC Test Strips



The fluorescence intensities generated on the 0.5 mg/ml antibody coated NC test strips were not as expected. With increasing conjugate concentration, the fluorescence intensity decreased until $1.47e^{-5}$ M concentration when the intensity increased again, and the blank strip generated a background intensity that was comparable to that of $3.74e^{-5}$ M conjugate test strip. Patonay saw reflected and scattered light from NC membranes with their detector system and it was probable that our observations were due to the same effect.[105] The slits and angle of detection had been optimised using Immunodyne ABC nylon test strips, these conditions were not suitable for NC membranes. This could be due to the structure of the NC, which is in effect a series of spheres immobilised together, whereas the nylon is a series of long strands.[180] Patonay experienced a maximum detrimental effect on the fluorescence

intensities when detected at 90° to NC that was improved by decreasing the angle to 64°. In our system using two optical fibres, one for the incident light transmission and one for returning the generated fluorescence to the detector could be used to reduce the angle of detection and light scattering effects. The angle between these fibres should be approximately 60°, but a decrease in fluorescence intensity may arise due to the inability of the fibres to get very close to the membrane surface. Therefore an optical arrangement where the incident light and detector are housed together would be the most practical.[105]

Figure 4.10 Concentration Curve Generated by Conjugate (52) on 0.05 mg/ml Antibody Coated Immunodyne ABC Nylon



The intensities obtained from the conjugate immobilised on the 0.5 mg/ml antibody coated nylon test strips increased with increasing concentration, the blank strip gave the lowest intensity. This result shows that the angle of detection was suitable for use with this membrane as the difference in conjugate concentration can be clearly detected, however, the intensity values seemed very high, which was attributed to the target slit being used (0.5, t, 0.5, 1.25). We decided to change the slits to 2.5, 2.5, 1.25, and 1.25 to bring the maximum intensity within the 1e5 - 9e6 cps ranges.

The emission spectra were repeated using $9.67e^{-6}$ M conjugate on nylon and Fluorotrans membranes using the new slit arrangement (Figure 4.11)

Figure 4.11 Fluorescence Intensities of Conjugate (52) on antibody Coated Nylon and PVDF Membranes

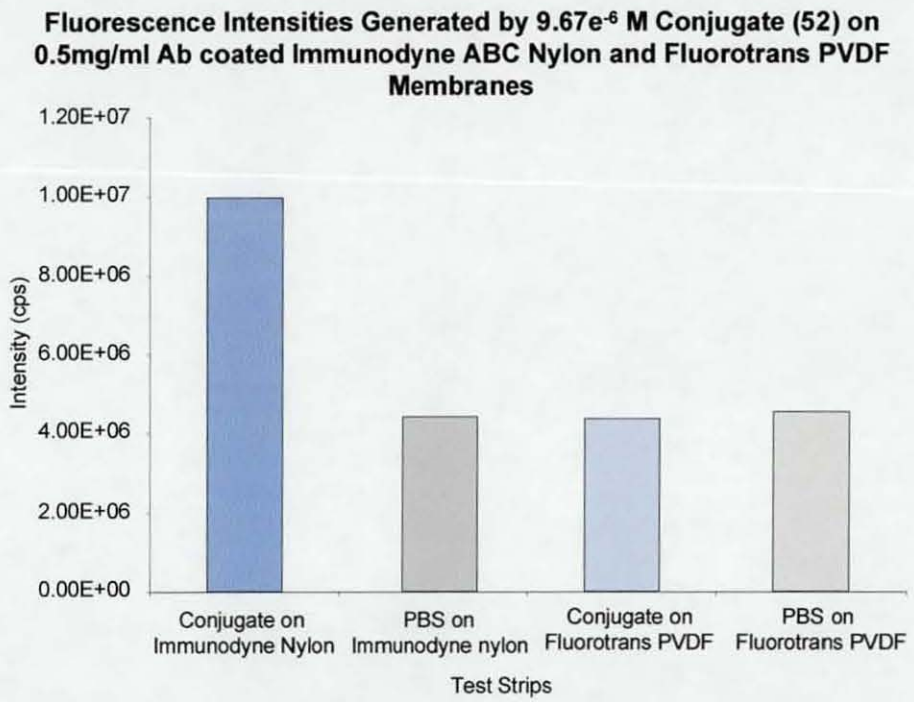


Table 4. 13 F/B Ratio for Conjugate (52) on NC, Nylon and Fluorotrans Strips

Millipore NC Nitrocellulose			Immunodyne ABC Nylon		
[(52)]	**I_f (cps)**	**F/B ratio**	**[(52)]**	**I_f (cps)**	**F/B ratio**
3.47E-05	4.92E+06	0.99	2.29E-05	1.24E+07	2.57
1.74E-05	4.40E+06	0.89	1.15E-05	1.22E+07	2.53
8.68E-06	4.53E+06	0.91	5.73E-06	9.40E+06	1.95
4.34E-06	4.51E+06	0.91	2.86E-06	9.01E+06	1.87
2.17E-06	4.80E+06	0.97	1.43E-06	5.31E+06	1.10
0.00E+00	4.96E+06		0	4.83E+06	
Fluorotrans new slits			**Immunodyne ABC new slits**		
9.67E-06	4.54E+06	1.04	9.68E-06	9.97E+06	2.25

Although the Immunodyne ABC nylon gave good fluorescence intensities, the Fluorotrans membrane gave no significant differences in fluorescence between the strips that were incubated in conjugate **(52)** and the blank

strips, which was probably due to the concentration of antibody immobilised on the strip. The Fluorotrans membrane is made from PVDF with a 0.2 μm pore size that should have a relatively large surface area, and therefore a higher antibody concentration, than the 0.45 μm Immunodyne ABC nylon membrane. However, the immobilisation of antibodies on PVDF membranes is related to the concentration of antibody in the incubation solution and also to the incubation times and temperatures, whereas the immobilisation of antibodies on the Immunodyne ABC nylon is instantaneous and only relies on the antibody concentration. Therefore, the antibody concentration on the Fluorotrans membrane may be too low to give any significant difference between the background and the immobilised conjugate **(52)**. We decided to continue exclusively with the Immunodyne ABC nylon membrane and optimise the immunoassay conditions accordingly.

4.8 Optimisation of Immunoassay Conditions

Assay design is composed of choosing the reagents and protocols that give the most accurate measurement of analyte concentration in the test sample in the shortest reasonable time.[175] A heterogeneous competitive immunoassay is to be developed that uses a limited concentration of antibody and labelled antigen and employs a reagent separation step. The inclusion of a separation step provides the opportunity for introducing a wash step, which has the advantage of improving the assay detection limits in situations where non-specific binding is crucial to the overall performance.[175] Therefore in the optimisation of immunoassay conditions several factors have to be considered namely, the antibody concentration, the blocking and washing conditions, and assay incubation time.

The solid surface in a heterogeneous immunoassay is selected for its ability to allow covalent attachments of antibodies and other proteins, so it is not unexpected that these surfaces are also prone to non-specific binding of the conjugate. This type of binding produces elevated signals in a uniform manner, regardless of the sample being tested and reduces the

ability of the assay to distinguish a true signal from the analyte from the background signal and reduces the assay sensitivity.

To prevent non-specific binding it was necessary to block the excess binding sites of the immunoassay solid phase. In an attempt to do this we added a solution of a non-cross reacting polymer (PVA) to the solid phase as an extra coating step once the antibody coating was complete. However, when this blocking agent was used with the nylon membrane a significant amount of non-specific binding still occurred, which could have given rise to erroneous results.

4.8.1 Addition of Detergents to Test Strip Manufacture

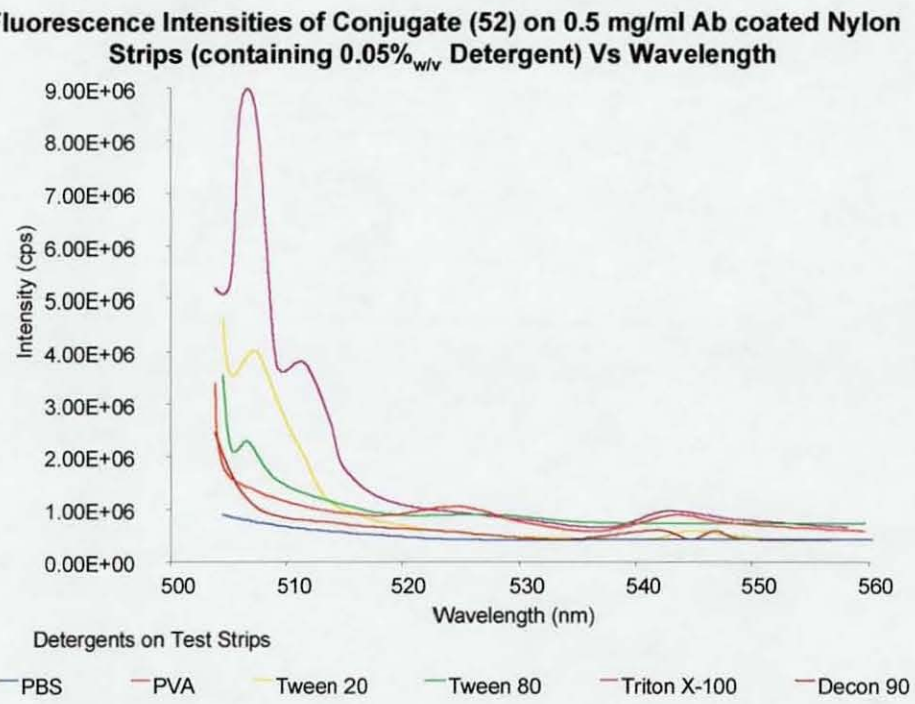
It was proposed by Place *et al.* that in an immunoassay, an acceptable response time of the order of minutes or less should be obtainable at μM antigen concentrations, however, lengthy response times will be found in the presence of a high degree of non-specific binding.[103] Several approaches were examined to eliminate, or substantially reduce, the non-specific binding of the conjugate to the nylon surface without increasing the immunoassay incubation times. For example, the additions of non-ionic detergents to the blocking solution during the test strip manufacture and addition of a blocking agent to the conjugate incubation solution.

Immunodyne ABC nylon test strips (0.5 mg/ml Ab) and blank strips were prepared, as described in Section 4.6.2, which contained detergents ($0.05\%_{w/v}$) in the PVA blocking solution (Table 4.14). The test strips were incubated in fluorescein-conjugate **(52)** (2 ml, $4.20e^{-6}$ M containing $1\%_{w/v}$ PVA) for 5 minutes, washed in Tween-20 (2 ml, 15 minutes), blotted on a tissue and an emission scan was performed using the parameters outlined in Table 4.12. The $1\%_{w/v}$ PVA strips were used as control strips and the fluorescence intensity was used to determine the effect of detergents on the non-specific binding. The fluorescence spectra are displayed in Figure 4.12 and the F/B ratio in Table 4.14.

Table 4. 14 Blocking Solutions used to Reduce Non-specific Binding (F/B Ratio for Conjugate (52) on Nylon Strips)

Blocking Solution	(I_f)	F/B ratio
$1\%_{w/v}$ PVA	$2.76e^5$	1.32
$1\%_{w/v}$ PVA + $0.05\%_{w/v}$ Tween-20	$1.29e^6$	6.17
$1\%_{w/v}$ PVA + $0.05\%_{w/v}$ Tween-80	$4.50e^5$	2.15
$1\%_{w/v}$ PVA + $0.05\%_{w/v}$ Triton x-100	$1.75e^6$	8.34
$1\%_{w/v}$ PVA + $0.05\%_{w/v}$ Decon-90	$2.26e^5$	1.09

Figure 4.12 Fluorescence Spectra of Conjugate (52) on 0.5 mg/ml antibody Coated Test Strips Containing 0.05% Detergents



The addition of detergents to the test strips had an unusual effect on the fluorescence intensities generated by the conjugate **(52)**. The Tween-20, Triton x-100 and Tween-80 coated antibody strips all gave enhanced fluorescence intensities, compared to the PVA control strips, and their emission maxima changed from 525 nm to 508 nm, reducing the Stokes' shift from 27 nm to 15 nm. Similar effects have been observed by Patonay *et al.* with their NC/milk/Tween-20 system[105] They suggested that a strong binding affinity existed between the Tween-20, dye conjugate

(51) and the NC, which was due to the hydrophobicity of the non-polar components in their system. With Triton x-100 an unexplained peak at 512 nm appeared and when Decon-90 was used no emission peak was detected in the 505 – 535 nm range. In all the conditions an additional peak appeared at 544 nm that had an intensity range of $1.50e^5$ cps to $1.0e^6$ cps. This was only seen when $1\%_{w/v}$ PVA was added into the conjugate solution and was probably due to Rayleigh scattering of the PVA solution. Bhattacharya *et al.* have shown that the spectral behaviour of dyes depends on their environment.[177] The varying fluorescent behaviour in different media may be related to the molecular configuration of the polymers (detergents) and/or the viscosity. It has been reported that the number of polyoxyethylene groups (the head group of Tween) in the detergent affects the efficiency of fluorescence, by reducing the polarity of the solution with increasing monomer residues, this would explain the difference observed between the Tween-20 and Tween-80 spectra..

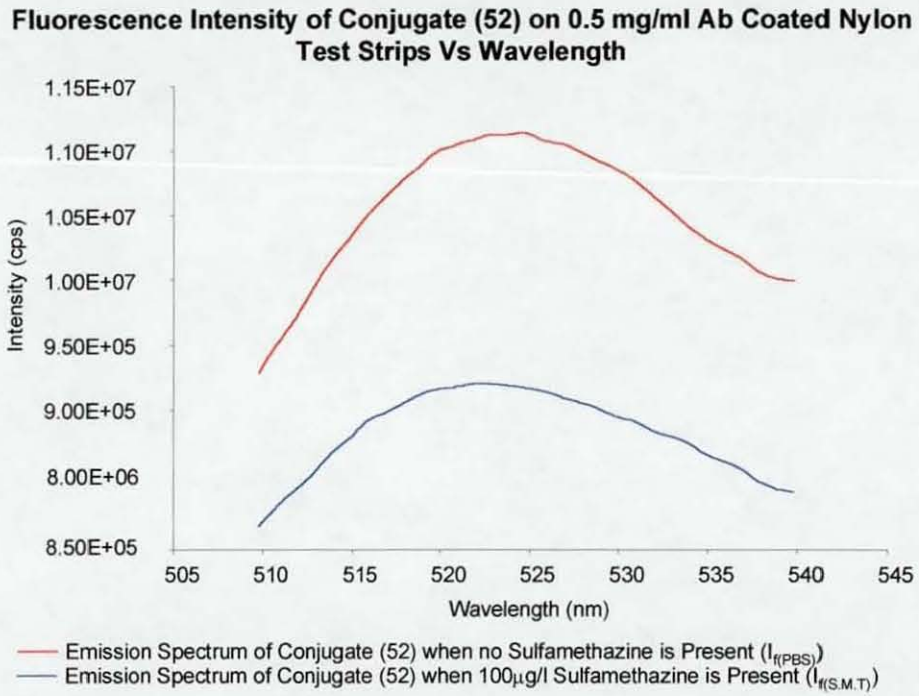
We decided to revert back to using conjugate **(52)** in PBS solution and $1\%_{w/v}$ PVA coated strips as the maximum fluorescence wavelength of the conjugate remained at 525 nm giving the largest Stokes' shift. The non-specific binding issue would be tackled by optimising the washing conditions if sulfamethazine could be detected.

4.8.2 Can Sulfamethazine Be Detected?

The reduction of the non-specific binding would be of no concern if sulfamethazine could not be detected on the test strip by the conjugate. We decided to run a competitive immunoassay where the sulfamethazine is incubated first because this has a greater antibody binding affinity than the conjugate **(52)**, which is used as a tracer. Test strips (0.5 mg/ml Ab) were prepared as described in Section 4.6.2 and were incubated in PBS or sulfamethazine (2 ml, 100 μg/l) for 30 minutes, then the conjugate **(52)** (2 ml, $8.129e^{-6}$ M) for 5 minutes. The strips were washed in Tween-20 solution (2 ml), for 15 minutes with orbital shaking, and blotted on a tissue. The intensity was determined by performing an emission scan using the parameters outlined in Table 4.12 and the results are displayed

in Figure 4.13 as a plot of Fluorescence Intensity (cps) Vs Wavelength (nm).

Figure 4.13 Emission Spectra Produced on Competition of Conjugate (52) With Sulfamethazine on Nylon Test Strips



A clear difference between fluorescence intensity of the negative sample ($I_{f(PBS)}$) and sulfamethazine positive solutions ($I_{f(s.m.t)}$) was seen. In light of this result a sulfamethazine concentration curve was produced, using smaller slits in order to bring the maximum emission intensity below $8e^6$ cps.

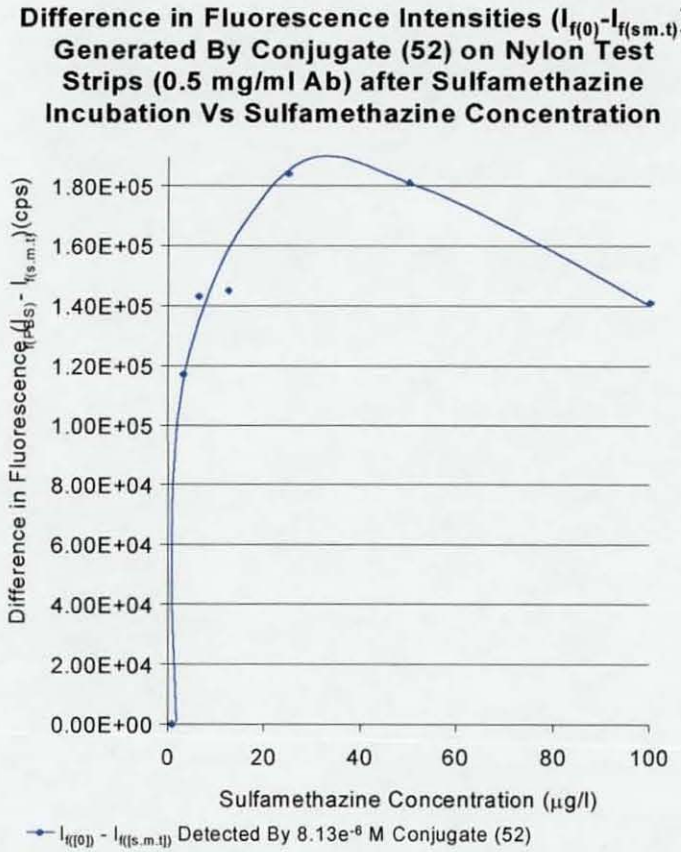
Test strips were prepared (0.5 mg/ml Ab) as described in Section 4.6.2. A series of sulfamethazine standards of 100, 50, 25, 12.5, 6.25 and 3.12 µg/l and a PBS blank were prepared described in Section 2.3.5. The test strips were incubated in the sulfamethazine standards (2 ml) and the conjugate **(52)** (2 ml, $8.129e^{-6}$ M) as described above and the intensity was determined by performing an emission scan using the following standard parameters (Table 4.15).

Table 4.15 Standard Fluorimeter Parameters Used to Produce an Emission Spectrum of Conjugate (52) on a Nylon Test Strip

Excitation	492.7 nm	**Emission**	500 – 600 nm	**Increment**	1 nm
Integration	0.5 s	**Slits – Excitation**		2.5, 2.5	
		Emission		1.25, 1.25	

The maximum fluorescence intensity was generated by the PBS incubated strip when the sulfamethazine concentration = 0 μg/l, ($I_{f([0])}$). The fluorescence intensity produced by the sulfamethazine incubated strip at each concentration, ($I_{f([s.m.t])}$), was subtracted from $I_{f([0])}$ to give the Difference in Fluorescence (cps). The Difference in Fluorescence (cps) Vs Sulfamethazine Concentration (μg/l) for the immunoassay is displayed in Figure 4.14.

Figure 4.14 Sulfamethazine Concentration Curve Detecting with Conjugate (52) on Nylon Test Strips (0.5 mg/ml Ab)



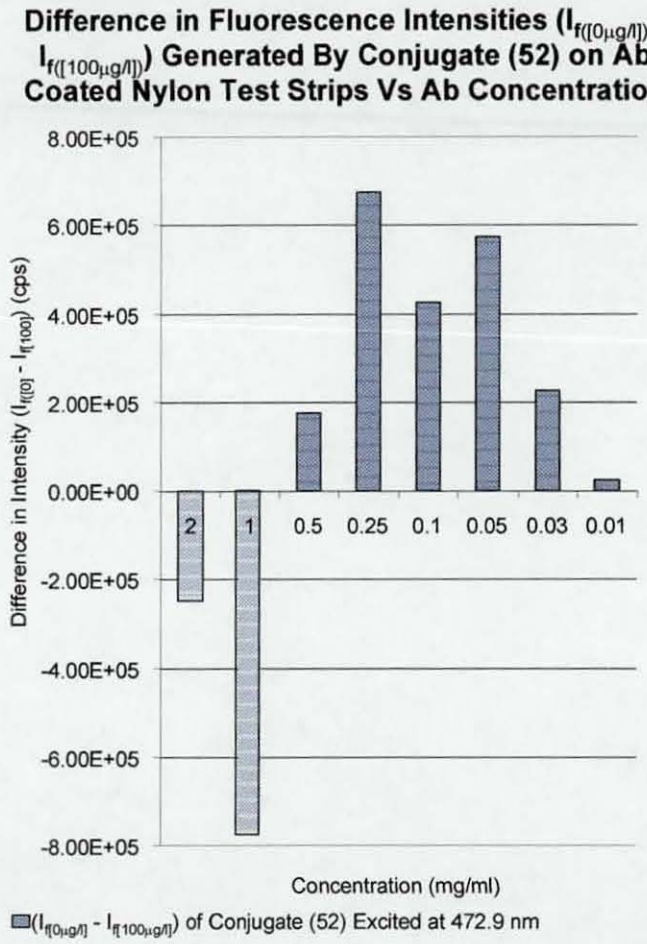
The difference in fluorescence intensity increased until approximately 40 μg/l then started to decrease again indicating the antibody or conjugate were too concentrated.

4.8.3 Optimisation of Antibody Concentration

Competitive assays are based on competition between the labelled antigen and the analyte in the sample for the binding sites on immobilised antibodies, to enable this competition a limited amount of coating antibody is used.[74]

In order to optimise the immunoassay antibody concentration a range of antibody concentrations (0.01 mg/ml to 2 mg/ml) was immobilised on to the Immunodyne ABC nylon test strips, which were prepared as outlined in Section 4.6.2. The strips were incubated in sulfamethazine (2 ml, 100 μg/l) or PBS solution (2 ml, 0 μg/l) then conjugate **(52)** (2 ml, $8.129e^{-6}$ M) as outlined in Section 4.8.2. The intensity was determined by an emission scan using the standard parameters (Table 4.15), and the results are expressed as Difference in Intensity (cps) Vs Antibody Concentration (mg/ml) in Figure 4.15.

Figure 4.15 Comparison of ($I_{f[0\mu g/l]} - I_{f[100\mu g/l]}$) Generated by Conjugate (52) With Decreasing Antibody Concentration



The determination of $I_{f([0])}$-$I_{f([100\mu g/l])}$ for conjugate **(52)** gave both positive and negative values; negative values were produced for 2 mg/ml and 1 mg/ml antibody coated strips because the fluorescence intensity generated when sulfamethazine was present was greater than when sulfamethazine was absent. This can be attributed to the antibody avidity and affinity for the conjugate. In the absence of sulfamethazine two conjugate molecules **(52)** can bind per bivalent antibody. The closeness of these bound fluorophores exhibits an inner-filter effect, distorting the fluorescence spectrum and reducing the fluorescence intensity. When the antibody was initially reacted with sulfamethazine, only one binding site was available for conjugate binding, which reduced the fluorophore crowding and increased the observed fluorescence. The differences that were seen between the 2 mg/ml and 1 mg/ml antibody test strips were consistent with this argument. With the 2 mg/ml antibody strip when all the

sulfamethazine was bound, a significant amount of antibodies were still available for binding to two conjugate molecules, which lowered the fluorescence intensity. Inner-filter effects were reported as a potential problem by San Román *et al.*[178] They found that these effects were a source of distortion of fluorescence spectra and lowered the observed luminescence quantum efficiencies of dyes adsorbed onto solid surfaces, and light scattering creates further complications as the efficiency of photon collection depends on the optical arrangement and absorbance of the sample.

Sadana and Chen established the theory that the binding of an antigen in solution to an antibody immobilised on a surface is a two-step process.[78] The elementary step involved the binding of a single antigen, over time t, and then the second antigen and had second-order reaction kinetics. They also described a situation where the reactants are spatially constrained on the microscopic level, this they suggest would give $^3/_2$ order kinetics, this was confirmed by Kopelman who stated that, non-homogeneous diffusion controlled reactions had time-dependent rate coefficients.[179]

The remaining antibody concentrations gave a decrease in fluorescence when competing against sulfamethazine, which was seen as a positive difference. The 0.5 mg/ml antibody concentration had previously shown a high dose hook (prozone effect) at higher sulfamethazine concentrations, which was due to the antigen saturating the antibody binding sites. This subsequently inhibits proportional binding of the second labelled antigen. It was decided to reduce the antibody concentration to 0.25 mg/ml as this gave the maximum difference of intensity in competition with sulfamethazine at this conjugate concentration.

4.8.4 Optimisation of Blocking Reagents and Wash Conditions

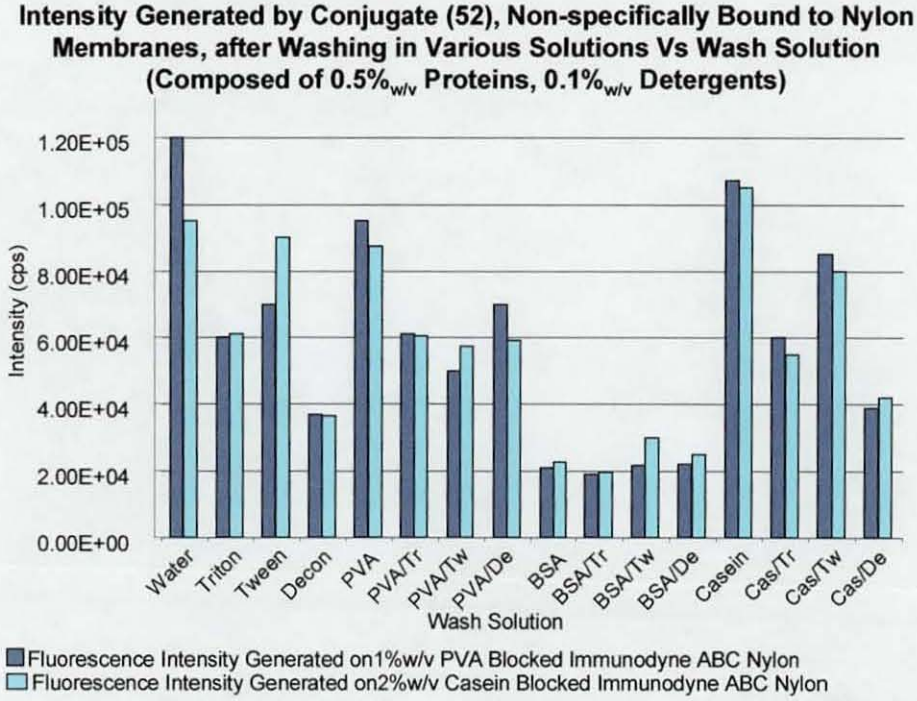
In order to separate the free from bound antigens and to reduce non-specific binding the assay wash conditions were investigated. Wash solutions were prepared in 0.1 M PBS (pH 7.4) using polymers, proteins and detergents as shown in the chequer-board arrangement (Table 4.16).

Table 4.16 Combinations of Polymers and Proteins ($0.5\%_{w/v}$) with Detergents ($0.1\%_{w/v}$) Used to Optimise Washing Conditions

	A	B	C	D
A'	H_2O	Triton x-100	Tween-20	Decon-90
B'	PVA	B + B'	C + B'	D + B'
C'	BSA	B + B'	C + C'	D + C'
D'	Casein	B + B'	C + D'	D + D'

Immunodyne ABC nylon test strips with no antibody were blocked with either PVA ($1\%_{w/v}$) or casein ($2\%_{w/v}$) and prepared as described in Section 4.6.2. These strips were incubated in conjugate **(52)**, (2 ml, $4.5e^{-6}$ M), for 5 minutes and washed for 15 minutes with orbital shaking using the wash conditions outlined above. The fluorescence intensity was determined by an emission spectrum using the standard parameters (Table 4.15) and displayed in Figure 4.16 as a plot of Fluorescence Intensity (cps) Vs Wash Solution.

Figure 4.16 Comparison of the Test Strip Washing Conditions for Nylon Strips, Fluorescence Generated by Conjugate (52)



It is seen that the majority of $2\%_{w/v}$ casein blocked test strips gave lower fluorescence intensities than the $1\%_{w/v}$ PVA blocked strips. This effect was probably due to the structure of Immunodyne ABC membrane, which is a chemically activated internally supported nylon 6,6 affinity membrane that will permanently immobilise proteins on contact at neutral pH.[180]

Washing with water gave the maximum observed intensity and is an indication of the total non-specific binding for this system. A shift in emission λ_{max} to 515 nm was seen with Tween-20 and Triton x-100 and a decrease in fluorescence with Decon-90, when the washing solutions contained the $0.1\%_{w/v}$ detergents alone. This was seen previously in Section 4.8.1. An increase in background fluorescence was seen with casein-blocked strips in the presence of Tween-20 and Triton x-100, except when casein was present in the wash solution. The addition of $0.5\%_{w/v}$ casein and PVA into the wash solutions did not significantly reduce the fluorescence intensity caused by non-specific binding. The best washing conditions were observed when $0.5\%_{w/v}$ BSA was incorporated into the wash solution. This gave the lowest background fluorescence indicating the minimum non-specific binding.

It has been suggested by Blake and Gould that antibodies may undergo denaturation on washing when non-ionic detergents are added to the test strip to prevent non-specific binding.[97] This has the effect of reducing both sensitivity and reproducibility of the assay, but they propose that the use of proteins covalently attached to the membrane surface should overcome these difficulties. In light of this, the overall choice of conditions employed to minimise non-specific binding was $2\%_{w/v}$ casein blocked test strips with a $0.5\%_{w/v}$ BSA + $0.1\%_{w/v}$ Triton x-100 in 0.1M PBS washing solution.

We noted that with these blocking and washing conditions the emission maxima shifted to 520 nm and the background fluorescence intensity was $2.00e^4$ cps. When this intensity was compared with the maximum intensity given by the water washing condition ($1.2e^5$), it is seen that the washing conditions reduce the non-specific binding by 17 %.

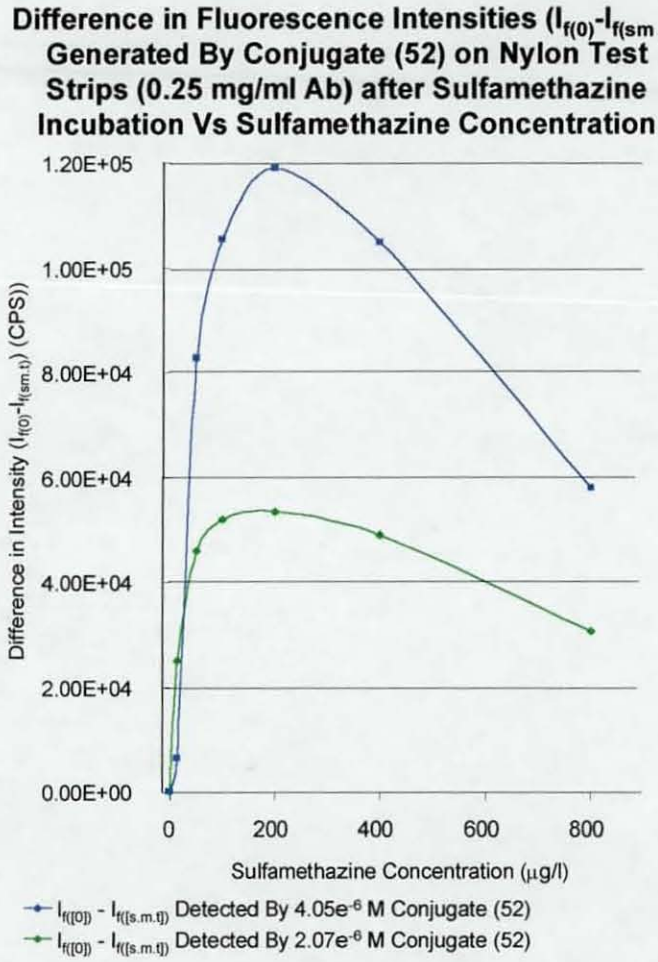
4.8.5 Competitive Assay of Sulfamethazine with Conjugate (52)

After optimising the blocking and washing conditions and reducing the antibody concentration a competition assay of sulfamethazine was performed. Antibody coated nylon test strips (0.25 mg/ml Ab), blocked with $2\%_{w/v}$ casein were prepared as outlined in Section 4.6.2 and a series of sulfamethazine standards with concentrations of 800, 400, 200, 100, 50, 25, 12.5 and 6.25 μg/l and a PBS blank were prepared as described in Section 2.3.5. The test strips were incubated for 5 minutes in the sulfamethazine standards (2 ml) then the conjugate **(52)** (2 ml, $4.05e^{-6}$ M) for 5 minutes. The strips were washed in $0.5\%_{w/v}$ BSA + $0.1\%_{w/v}$ Triton x-100 in PBS washing solution (2 ml, 15 minutes) with orbital shaking, blotted on a tissue, and the fluorescence intensity was determined by an emission spectrum using the standard parameters (Table 4.15). The results are displayed in Figure 4.17 as a plot of Difference in Fluorescence Vs Sulfamethazine Concentration (μg/l).

As the sulfamethazine concentration increased the difference in fluorescence increased as expected until 200 μg/l. At this concentration the intensity started to decrease again giving a prozone effect, which was due to either the antibody or conjugate being too concentrated. The prozone effect would have effectively reduced the assay working range and given rise to false positive results.

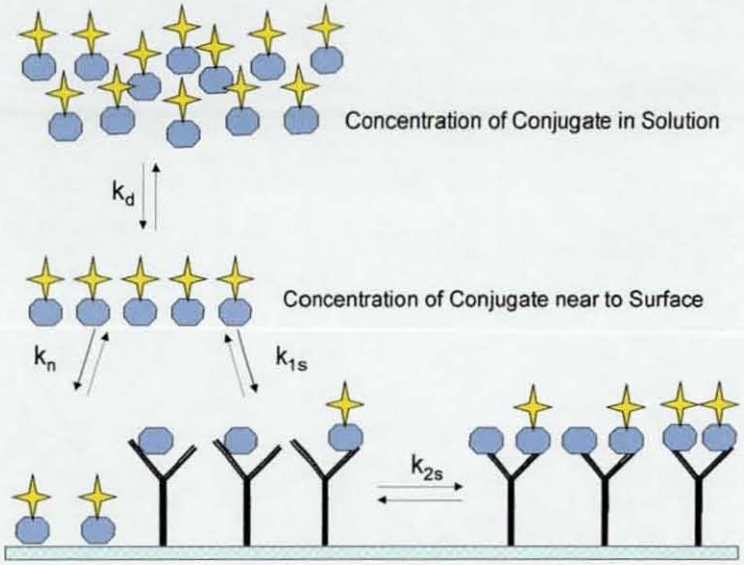
The conjugate concentration was decreased to $2.07e^{-6}$ M and the immunoassay was repeated using the conditions as outlined above. The results are also displayed in Figure 4.17.

Figure 4.17 Sulfamethazine Concentration Curve Detecting with Conjugate (52) on Nylon Test Strips (0.25 mg/ml Ab)



With the lower conjugate concentration (2.07e^{-6} M), the overall fluorescence intensities had decreased but the same prozone trend was noticed. The fluorescence intensities generated by the more concentrated conjugate (4.05e^{-6} M) were attributed to the non-specific binding and diffusion effects as proposed by Sadana and Chen in their theoretical model. [77] They proposed that the non-specific and specific binding are an effect of concentration, are diffusion controlled, and that the presence of non-specific binding influences the diffusion-controlled specific binding of antigen in solution to antibody immobilised on a surface (Figure 4.18).

Figure 4.18 Representation of Diffusion Controlled Specific Binding of Antigens in Solution to Antibodies on a Surface[77,78]



The concentration of the conjugate in bulk solution diffuses to the surface with a rate of k_d. The concentration of conjugate at the surface controls its binding to the antibodies immobilised on the surface. Sadana and Chen are proposing that the non-specific binding (k_n) is a faster reaction than the specific antibody binding (k_{1s}) and as such, the non-specific binding controls the concentration of conjugate at the surface and thus the specific binding.

The lower concentration conjugate solution would have given a reduced concentration at the solid surface generating less non-specific binding. As the non-specific binding is controlling the rate-determining step of the overall binding reaction, less specific binding of the conjugate could occur, which was seen as a reduction in the fluorescence intensity. This result also re-enforces the idea that the reaction was not a first-order reaction (Section 4.8.3), as the fluorescence intensity was related to both concentration and time.

To reduce the prozone effect and produce an assay with a working range of 0 – 1000 μg/l the antibody concentration was reduced to 0.10 mg/ml.

4.8.6 Optimisation of Immunoassay Wash Times

Some elution of bound antigen from the solid phase invariably occurs during washing and incubation steps. For this reason, the time and manner of the washing and incubations steps must be carefully standardised for reproducible results.[74]

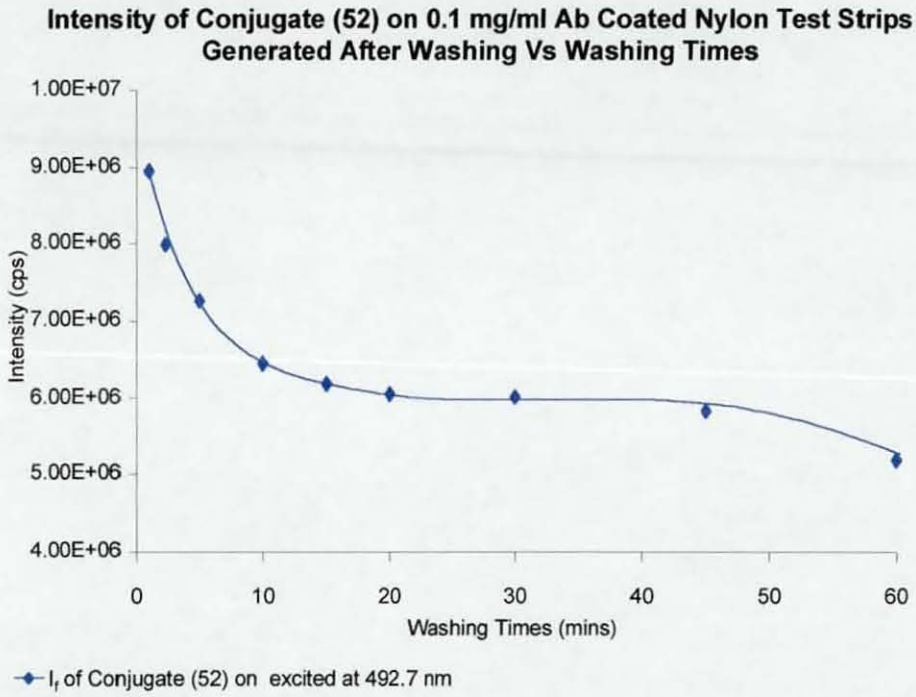
Although the washing solution was optimised to give minimum non-specific binding, until now an arbitrary time of 15 minutes has been used. The effect of increasing the washing time on the fluorescence intensity of the assay has already been noticed in Section 4.8.1, so it was necessary to optimise the washing times for the assay. The times tested are given in Table 4.17.

Table 4.17 Optimisation of the Immunoassay Wash Times

Time	Average Intensity	% CV
1	$8.960e^{6}$	3.85
2	$7.947e^{6}$	2.77
5	$7.260e^{6}$	1.91
10	$6.450e^{6}$	0.98
15	$6.075e^{6}$	1.97
20	$6.045e^{6}$	3.46
30	$6.010e^{6}$	0.78
45	$5.614e^{6}$	4.71
60	$5.194e^{6}$	3.23

Ab coated strips (0.10 mg/ml) blocked with casein ($2\%_{w/v}$) were prepared as outlined in Section 4.6.2. The test strips were incubated for 5 minutes in a PBS blank solution (2 ml) then the conjugate **(52)** (2 ml, $2.70e^{-5}$ M) for 5 minutes. The strips were washed with $0.5\%_{w/v}$ BSA + $0.1\%_{w/v}$ Triton x-100 in 0.1M PBS washing solution (2 ml), for x minutes with orbital shaking, and blotted on a tissue. The fluorescence emission intensity was determined using the standard fluorimeter parameters (Table 4.15) and the results are displayed in Figure 4.19 as a plot of Fluorescence Intensity (cps) Vs Time (min).

Figure 4.19 Optimal Immunoassay Washing Times



As the wash time increases the fluorescence intensity of conjugate **(52)** decreased levelling out between 15 and 45 minutes then decreasing again. The precision of the readings were calculated and were expressed as % CV, these varied from 0.78 to 4.71%. The probable cause for the variations in % CV was either residual Triton x-100 being present on the strip, or inconsistent blotting leading to variations of strip wetness.

Wetness of the strip was found to be problem by Patonay *et al.*[105] This problem was overcome by air-drying their strips and measuring the fluorescence intensity after 2 hours. Drying the strips reduces the quenching effect of water and produces a stable fluorescence signal that depends only on the dye and its immediate environment.

The washing time chosen for the immunoassay was 15 minutes as there was no significant change in the fluorescence intensity for the following 30 minutes.

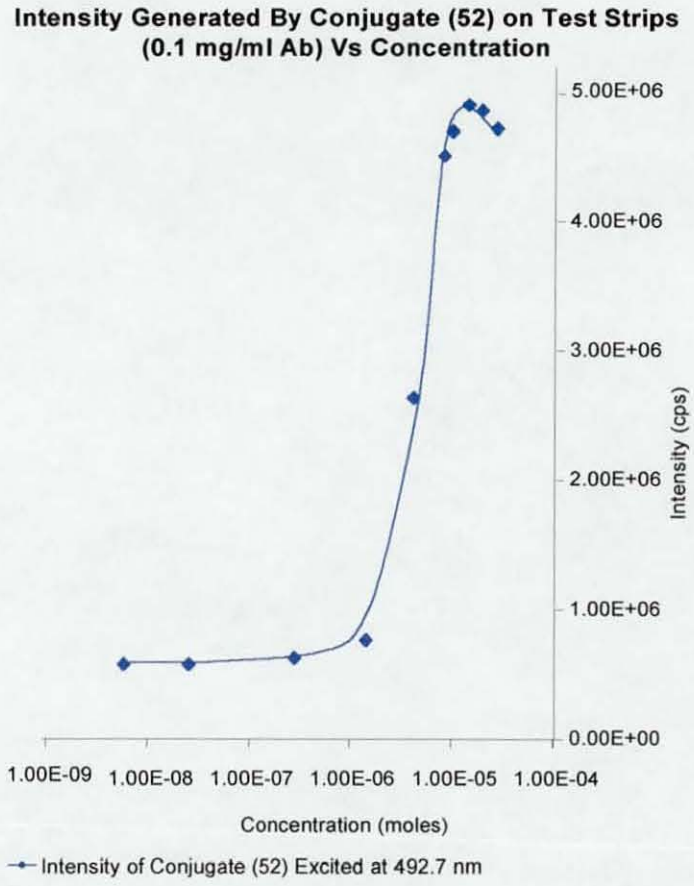
4.8.7 Optimisation of Conjugate Concentration

It has been reported that it is the excess reagent (non-competitive) assays systems that are most influenced by non-specific binding. By contrast the

performance of a limited reagent (competition) assay is determined more by the equilibrium constant of the reagent-antibody binding.[175]

The concentration of conjugate to be used in the immunoassay was optimised using 0.1 mg/ml antibody coated, $2\%_{w/v}$ casein blocked test strips prepared as described in Section 4.6.2. The test strips were incubated for 5 minutes in the conjugate solution **(52)** (2 ml, $2.82e^{-6}$ M to $5.80e^{-9}$ M), washed in $0.5\%_{w/v}$ BSA + $0.1\%_{w/v}$ Triton x-100 washing solution (2 ml), for 15 minutes with orbital shaking, and blotted on a tissue. The fluorescence intensity was determined using the standard parameters (Table 4.15). The results are expressed in Figure 4.20 as a plot of Fluorescence Intensity (cps) Vs Conjugate Concentration (moles) and in Table 4.18 as the Conjugate : Antibody ratio

Figure 4.20 Concentration Curve of Conjugate (52) on 0.1 mg/ml Antibody Coated Test Strips



Maximum fluorescence intensity was seen when $1e^{-5}$ M conjugate was present in the solution, which was equivalent to $6.05e^{-7}$ M conjugate on the test strip and an antibody to conjugate ratio of 1 : 0.92 (Table 4.18).

Table 4. 18 Ratio of Conjugate to Antibody Concentrations

[(52)] M in solution	[(52)] M on Strip	Conjugate : Antibody ratio
$2.82e^{-5}$	$1.71e^{-6}$	2.56
$2.03e^{-5}$	$1.23e^{-6}$	1.84
$1.47e^{-5}$	$8.89e^{-7}$	1.33
$1.00e^{-5}$	$6.05e^{-7}$	0.92
$9.97e^{-6}$	$6.01e^{-7}$	0.905
$7.37e^{-6}$	$4.46e^{-7}$	0.67
$3.69e^{-6}$	$2.23e^{-7}$	0.34
$1.47e^{-6}$	$8.89e^{-8}$	0.13
$2.95e^{-7}$	$1.78e^{-8}$	$2.7e^{-2}$
$2.59e^{-8}$	$1.57e^{-9}$	$2.4e^{-3}$
$5.80e^{-9}$	$3.51e^{-10}$	$5.3e^{-4}$

There are two binding sites for sulfamethazine on each antibody therefore a 1:2 conjugate to binding site ratio was seen. When this ratio was exceeded the fluorescence intensity decreased, which is consistent with the inner-filter effect argument raised in Section 4 8.3.

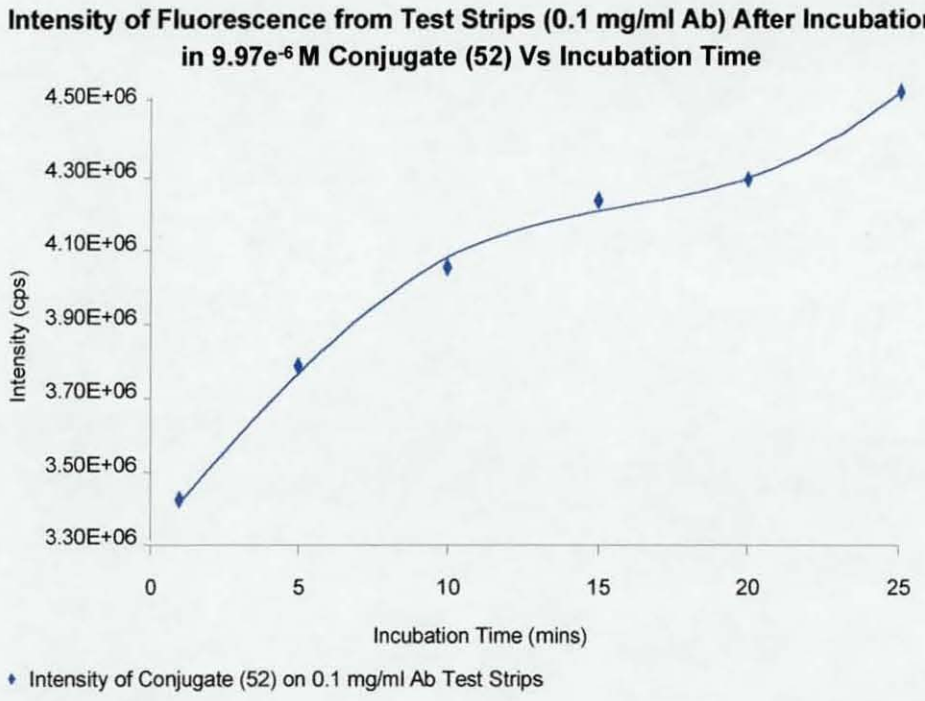
The remaining assay development was performed using $9.97e^{-6}$ M conjugate as this concentration gave high fluorescence intensity and an UV absorbance of 0.300 that allowed accurate preparation of standard solutions.

4.8.8 Evaluation of Assay Incubation Time

The assay time is dependent on the affinity of the antibodies for the conjugate and the incubation temperature, which will affect the time taken to reach equilibrium. The optimal incubation time was investigated using 0.1 mg/ml antibody strips and $9.97e^{-6}$ M conjugate **(52)**.

The test strips were prepared as described in Section 4.6.2 and incubated from 1 to 25 minutes in conjugate **(52)**, (2 ml, $9.97e^{-6}$ M). The strips were washed in $0.5\%_{w/v}$ BSA + $0.1\%_{w/v}$ Triton x-100 washing solution (2 ml), for 15 minutes with orbital shaking, blotted on a tissue and the fluorescence intensity was determined using the standard parameters outlined in Table 4.15. The results shown in Figure 4.21 and expressed as a plot of Intensity (cps) Vs Time (min).

Figure 4.21 Changes in Fluorescence with Increasing Incubation Time



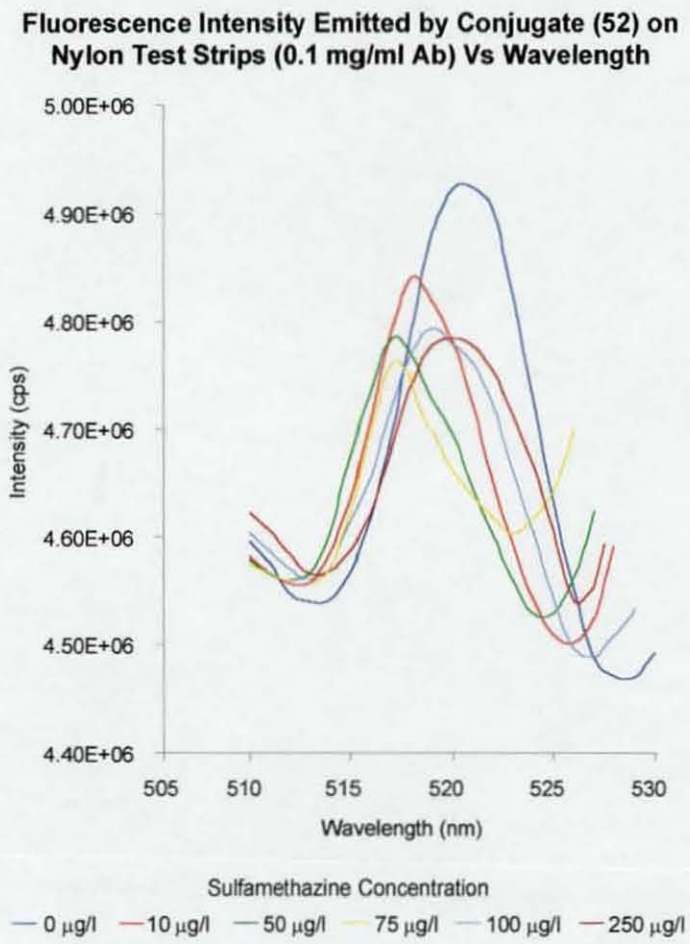
After 25 minutes the observed fluorescence intensity was still increasing, as the conjugate-antibody binding had not reached equilibrium. This occurred because to the antibody binding was diffusion controlled and is temperature dependent. Patonay *et al* found that the best performance for their near infrared immunoassay was 2.5 hrs, which included the test strip drying time.[105]

However, for our immunoassay an incubation time greater than 25 minutes was considered to be too lengthy so the original incubation time of 5 minutes was used.

4.8.9 Complications

The test strips (0.1 mg/ml Ab, 2%$_{w/v}$ casein) were prepared as in Section 4.6.2 using a new batch of 0.45 μm pore Immunodyne ABC nylon, and a series of sulfamethazine standards of 250, 100, 75, 50, 10 and 0 μg/l were prepared as in Section 2.3.5. An immunoassay was run with a 5 minute sulfamethazine incubation (2 ml) followed by 5 minutes conjugate incubation (2 ml, 9.97e-6 M) and 15 minute wash in 0.5% BSA + 0.1% Triton x – 100 wash solution. The emission spectra were obtained using the standard parameters outlined in Table 4.15 and are displayed in Figure 4.22.

Figure 4.22 Emission Spectra of Conjugate (52) on Test Strips (0.1 mg/ml Ab) in the Presence of Sulfamethazine



Several problems in the immunoassay results were seen when these assay conditions were used. A variable shift in the maximum emission wavelength for the conjugate in the presence of sulfamethazine was seen, which could have been due to wash solution remaining on the test strip. The intensity of fluorescence decreased as expected until 50 μg/l but then flattened out to give an undulating pattern instead of decreasing. We thought that these erroneous results could have been caused by the target area being touched by an un-gloved hand, or the use of a new batch of Immunodyne ABC, or sulfamethazine being transferred to the conjugate solution during incubation procedure; these effects were investigated.

It was noticed that until now powderless gloves had been used, however, during this experiment powered latex gloves were worn. It was discovered that the talc in the gloves gave emission peaks at 510, 518, 520 and 550 nm when excited at 497.2 nm, and the latex itself exhibited peaks at 519 and 530 nm, so the use of powderless nitrile gloves was re-established.

Although membrane batch-to-batch variations are known to occur, changing the Immunodyne ABC membrane had no significant effect on the assay.[180] Nevertheless, it was decided to continue with the original batch of membrane until a working assay was attained.

Thorough washing steps are necessary to ensure no carry-over of reagents from one step to the next, so an additional wash step was incorporated into the assay procedure.[74]

4.9 New Assay Procedure

After optimising the parameters several changes were made to the new assay procedure: Firstly the area of the test strip was reduced to 0.64 cm^2, this conserved membrane material yet retained an area large enough to use the fibre optics.

Secondly the volume of the incubation solutions were reduced to 100 μl. A series of volumes, 250 μl – 50 μl, were tested for their ability to remain in one defined area on the membrane surface. It was decided that 100 μl gave the best membrane coverage without the globule rupturing. This was pipetted directly onto the test strip target area using a 200 μl Gilson pipette. This decision was made because the assay was mainly diffusion controlled, and only the conjugate at the solid-phase surface is involved in the antibody binding reactions, so to have a large reservoir of conjugate which was not taking place in the reaction seemed wasteful. Moreover, the assay was to be adapted for use with other dye-conjugates that were in limited supply so a minimal volume of conjugate was used.

Thirdly an additional wash step was added. It was thought that the addition of a one-minute wash in water would improve the precision of the assay as well as reduce the chance of sulfamethazine being transferred into the conjugate incubation solution.

To maximise the assay precision and sensitivity it was necessary to exercise great care to pipetting and other reagent manipulation steps.

4.9.1 Test Strip Preparation

Pall Immunodyne ABC Nylon membrane was cut into 0.8 cm x 7.5 cm strips, stuck onto Autostat heat stabilised polyester cards using double-sided Scotch™ tape and the surface of the membrane scored into 0.64 cm^2 squares (0.8 cm x 0.8 cm). The fixed membranes were immersed in an antibody solution (50 ml, 0.10 mg/ml) and incubated at 30°C for 30 minutes. The cards were dried (30°C, 30 minutes), blocked by immersion in casein (2%$_{w/v}$, 22°C, 30 minutes), sucrose coated (3%$_{w/v}$, 30°C, 30

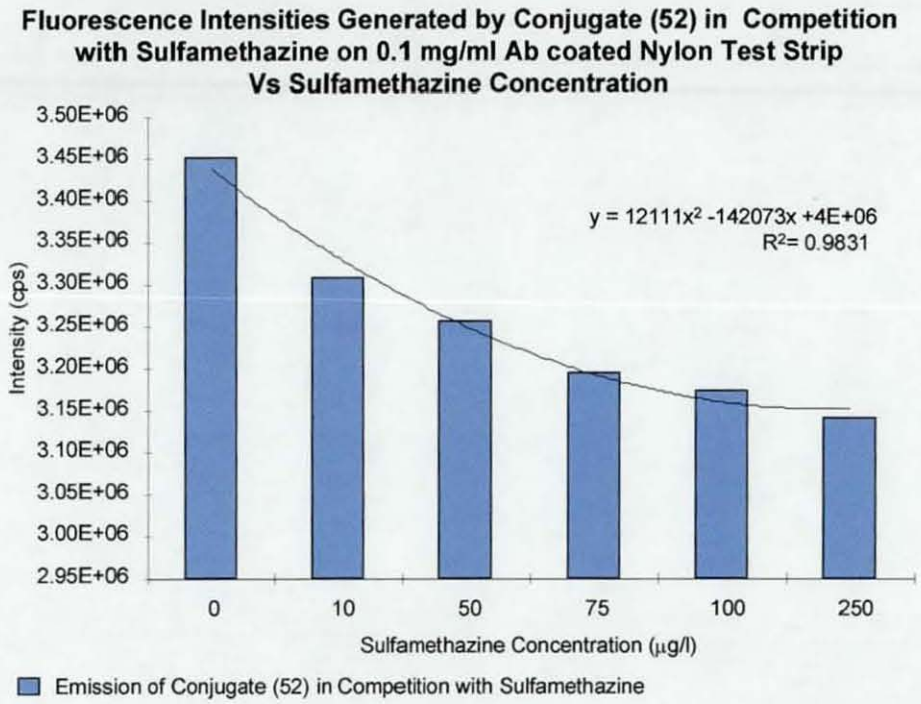
minutes), dried (30°C, 30 minutes) and cut into individual strips, giving one test per strip. These were stored in desiccated pots for up to 3 months.

4.9.2 Competitive Assay of Sulfamethazine with Conjugate (52)

The new procedure for the competitive immunoassay is outlined below. The test strips were prepared as outlined in Section 4.9.1 and series of sulfamethazine standards of 250, 100, 75, 50, 10 and 0 μg/l were prepared as described in Section 2.3.5. The test strips were incubated for 5 minutes in the sulfamethazine standards (100 μl) then washed in water for 1 minute with shaking. The strips were removed, blotted and then incubated with the conjugate **(52)** (100 μl, $9.97e^{-6}$ M) for 5 minutes. Next the strips were washed in $0.5\%_{w/v}$ BSA + $0.1\%_{w/v}$ Triton x-100 washing solution (2 ml), for 15 minutes with orbital shaking, and blotted on a tissue.

The fluorescence intensity was determined by an emission spectrum using the standard parameters (Table 4.15), and the results are displayed in Figure 4.23 as a plot of Fluorescence Intensity (cps) Vs Sulfamethazine Concentration (μg/l).

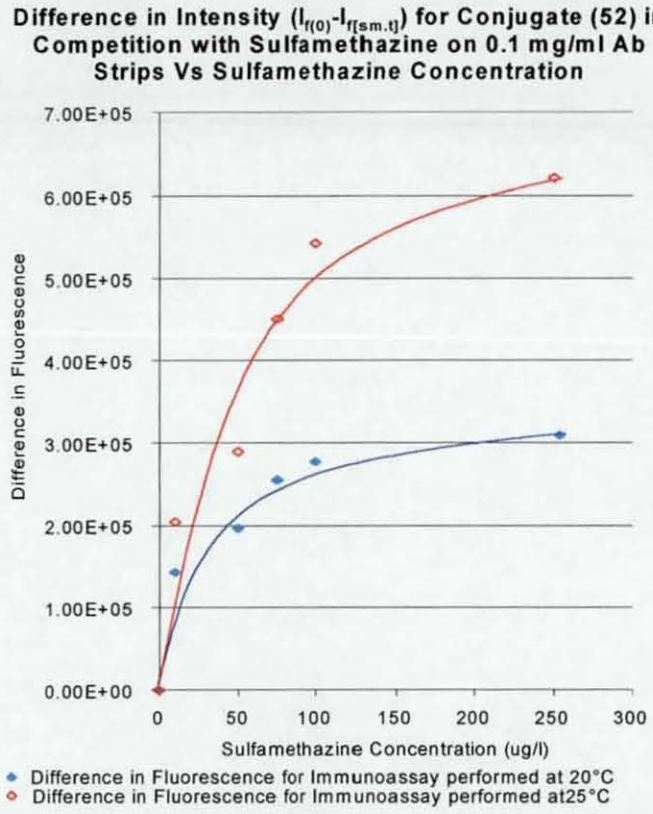
Figure 4.23 Competition Assay of Conjugate (52) and Sulfamethazine on 0.1 mg/ml Antibody Coated Test Strips



As the concentration of sulfamethazine increased the fluorescence intensity of the conjugate on the strips decreased, with no evidence of a prozone effect, giving a working assay that could detect the presence of sulfamethazine. The sensitivity of the assay was defined as the lowest concentration that could be detected which was not zero, which in this system was 10 µg/l. The sensitivity also relies on precision of individual measurements at or close to zero, and the precision for this assay was 1.25 - 1.51%.

The assay was repeated at 20°C and 25°C using the new assay protocol and the standard fluorimeter parameters outlined in Table 4.15. A plot of Difference in Fluorescence Vs Sulfamethazine Concentration (µg/l) was created for the results produced at these temperatures (Figure 4.24)

Figure 4.24 Sulfamethazine Concentration Curve Detecting with Conjugate (52) on Nylon Test Strips (0.10 mg/ml Ab)



It was seen that when the assay was performed at the higher temperature a larger difference in fluorescence intensities were seen and a steeper dose-response curve was generated.*** This was expected as the antibody-antigen equilibrium is known to be temperature dependent (Equations 4.1)

$$\underset{\text{(Free fraction)}}{Ag + Ab} \underset{k_d}{\overset{k_a}{\rightleftharpoons}} \underset{\text{(Bound fraction)}}{Ag\text{-}Ab} + \underset{\text{(Tracer)}}{Ag^*} \underset{k_d}{\overset{k_a}{\rightleftharpoons}} \underset{\text{(Bound fraction)}}{Ag\text{-}Ab\text{-}Ag^*}$$

Equation 4.1

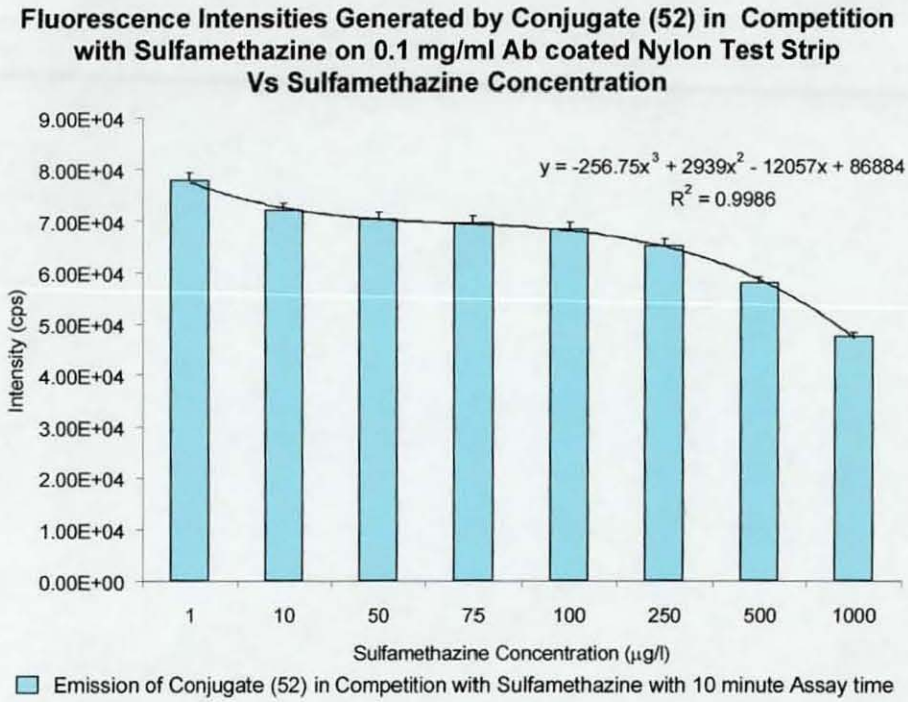
*** With increasing temperature the rate of dissociation from the antibody will increase. This would lead to a decrease in sulfamethazine concentration after test strip incubation, which would be seen as an increase in fluorescence as more antibody sites would be available for fluorescent conjugate binding. In this experiment the opposite effect is seen. The author proposes that this effect was due to the temperature dependency of the fluorophore; as the temperature increases the quantum efficiency of the fluorophore would decrease. This reduction in fluorescence could be exaggerated due to the fluorophore being immobilized onto a solid surface and would give the trend observed above. The author also proposes that with this degree of temperature dependence it is unlikely that the fluorescein immunoassay would be of use in a commercial setting.

For two assays with the same level of precision, the assay with the steepest dose-response curve is the most sensitive, which in our example was found at 25°C.

4.9.3 Reduction of Assay Time

The reduction in assay time for the new assay procedure was investigated. The test strips (0.10 mg/ml Ab, 2%$_{w/v}$ casein) were prepared as outlined in Section 4.9.1 and the sulfamethazine standards of 1000, 500, 250, 100, 75, 50, 10 and 0 μg/l were prepared as described in Section 2.3.5. The test strips were incubated for 2 minutes in the sulfamethazine standards (100 μl) and washed in water for 1 minute with shaking. The strips were removed, blotted and incubated with the conjugate **(52)** (100 μl, 9.97e^{-6} M) for 2 minutes, washed in 0.5%$_{w/v}$ BSA + 0.1%$_{w/v}$ + Triton x-100 wash solution (2 ml, 5 minutes) with orbital shaking, blotted on a tissue and the fluorescence intensity determined using the standard parameters (Table 4.15). The results are displayed in Figure 4.25 as Fluorescence Intensity (cps) Vs Sulfamethazine Concentration (μg/l).

Figure 4.25 Competition Assay of Conjugate (52) and Sulfamethazine on 0.1 mg/ml antibody Coated Test Strips with a 10 minute Assay Time



The overall fluorescence intensity was reduced by 97.7% compared with the longer assay time, however, a detectable difference between the sulfamethazine concentrations was determined and no prozone effect was seen. The precision at each sulfamethazine concentration was good, but the gradient of the slope does not significantly increase until 100 µg/l. Thus the 10 minute assay was regarded as less sensitive than the 26 minute assay having a lowest detectable dose of 100 µg/l.

4.9.4 Other Considerations

Specificity is defined in terms of the ability of an assay to produce a measurable response only for the antigen of interest. In immunoassays this discriminatory response is the result of the inherent nature of the immune system, that can produce an almost limitless variety of binding sites on the antibodies, and have a very strong affinity for specific chemical structures or parts of structures of an antigen. Since similar antigenic determinants may exist within other molecules there is a

possibility of an antibody having cross-reactivity. Cross-reactivity is dependent upon the quality of the antisera used, which in turn is partly dependent on the purity of the immunogen used to raise the antibody.

Roy Jackman of the Veterinary Laboratories Agency, Weybridge, provided the anti-sulfamethazine antiserum used throughout the research, which was raised in Soay sheep by immunisation with sulfamethazine-ovalbumin immunogen and Freund's complete adjuvant. It had a cross-reactivity with various other sulfa-drugs particularly sulfamerazine and sulfaquinoxaline (Appendix A.4.2).[75]

When these sulfa-drugs are present in the biological sample, they will compete with sulfamethazine for the antibody and produce a significant decrease in the conjugate fluorescence, which would be interpreted as a high sulfamethazine concentration, giving a false positive result. Thus the accuracy of the immunoassay would depend on minimising or eliminating the cross-reactive components. This could be achieved by increasing the incubation time and temperature of the immunoassay, but the success of these increases is dependent on the temperature affecting the sulfamethazine binding equilibrium distinctly from the other cross-reactants, and the sulfamethazine having a slower rate of dissociation.[92]

4.10 Near Infrared Dyes

Fluorescence spectroscopy has long been established as a sensitive and easy to use analytical technique, such as chromatography and immunoassays.[126,76] Several types of fluorescent molecules are suitable for labelling a wide range of analytes, for example, the fluorophores fluorescein, rhodamine, and Texas Red. These visible fluorophores were the most commonly used fluorescent labels until the advent of the cyanine dyes.[172] The use of far visible and near infrared cyanine dyes has been investigated in this study. It was anticipated that these dyes would avoid the interferences by biomolecules that can occur in visible spectral region. The symmetrical pentamethine cyanine dye, Cy5.18.OH **(35)**, was the first fluorophore to be chosen as it has a λ_{max} at 650 nm and ε of 200,000 and

it was reported to be bright, water-soluble and easily converted to the succinimidyl ester for conjugation to primary amines[138]

4.10.1.1 Pentamethine Cyanine Dye Conjugates

The near infrared dye conjugates chosen for this study were based on the indirect labelling (using proteins) and direct labelling of sulfamethazine **(1)** with Cy5.18.OH **(35)**. It was predicted that the direct linkage of Cy5.18.OH **(35)** and sulfamethazine **(1)** would decrease ε of the dye and reduce the antibody specificity of the sulfamethazine group. Therefore the addition of a spacer group in the conjugate structure was designed to prevent these effects. Jackman *et al.* found that the addition of an alkyl chain spacer, containing 8 or more carbons, between the sulfamethazine and fluorophore is ideal.[181] This distance would be long enough to prevent any electron withdrawing effects on the fluorophore and enable antibody binding.

The indirect labelling of sulfamethazine **(1)** and Cy5.18.OH **(35)** used horseradish peroxidase (HRP) as a linker and produced conjugate **(54)**. Conjugate **(37A)** was prepared as outlined in Section 3.2.1 and as discussed previously in Section 4.1.1. The sulfamethazine conjugate **(37A)** was reacted with Cy5.18.OSucc **(47)** to give conjugate **(54)**. The mode of reaction was not known, although, two processes could have occurred. Firstly, as the succinimidyl ester is a good leaving group, the ester reacted with the alcohol groups to form another ester; alternatively, the succinimidyl ester group reacted with the primary amine group of the lysine residues found in HRP or the diamine groups added during **(37A)** synthesis, giving a peptide.

Conjugate **(54)** was formed which had a 1:1:2 dye:protein:sulfamethazine ratio, which showed activity towards sulfamethazine-antisera, and gave a fluorescence spectrum in the 650 nm region. Unfortunately, conjugate **(54)** exhibited the same non-specific binding as the conjugate **(37A)** starting material so the investigations were taken no further.

In the next synthesis, conjugate **(55)**, was made by reacting Cy5.18.OSucc **(47)** directly with sulfamethazine **(1)**, to give an amide in 14% yield (Section 3.4.4). The molar absorptivity was reduced to 44,000 $M^{-1}cm^{-1}$ from 200,000 $M^{-1}cm^{-1}$, and the problem of low antibody specificity was observed. The conjugate was 28 times less active than sulfamethazine alone so investigations of conjugate **(55)** was taken no further.

Conjugates **(56)** and **(58)** were prepared by the same method outlined below (Sections 3.4.5 and 3.4.7). Cy5.18.OSucc **(47)** was activated with EDC and the sulfamethazine-linker compounds **(38)** and **(40)** were added to give conjugates **(56)** and **(58)**, respectively. The molar absorptivity of conjugate **(56)** was reduced to 46,000 $M^{-1}cm^{-1}$ and had an antibody specificity of 82 times less active than sulfamethazine alone. The reaction to form **(58)** gave two products, the hydrated Cy5.18.OH **(35)** and a conjugate that was not soluble in water. No further tests were performed on these conjugates.

In the synthesis of conjugate **(59)**, the carboxylic acid groups of Cy5.18.OH **(35)** and sulfamethazine-linker **(39)** were activated with EDC followed by the introduction of diaminooctane. Due to purification problems we only isolated a poor yield of the desired product and many other products that were detected by TLC. The conjugate **(59)** was tested by ELISA and found to be 16 times less active than sulfamethazine alone, so no further experiments were performed.

The formation of conjugate **(60)** was carried out in HMPT as this is miscible with both organic and aqueous solvents. This was important because the Cy5.18.OH **(35)** was only soluble in highly polar solvents. The reaction between Cy5.18.OH **(35)** and sulfamethazine-linker **(42)**, which has a bromo-leaving group, took place in the presence of sodium hydroxide and formed an ester. The product formed had a molar absorptivity of 89,000 $M^{-1}cm^{-1}$ and specificity 1.66 times less active than sulfamethazine. This conjugate **(60)** gave the best antibody specificity and molar absorptivity to date and was therefore used in the immunoassay development.

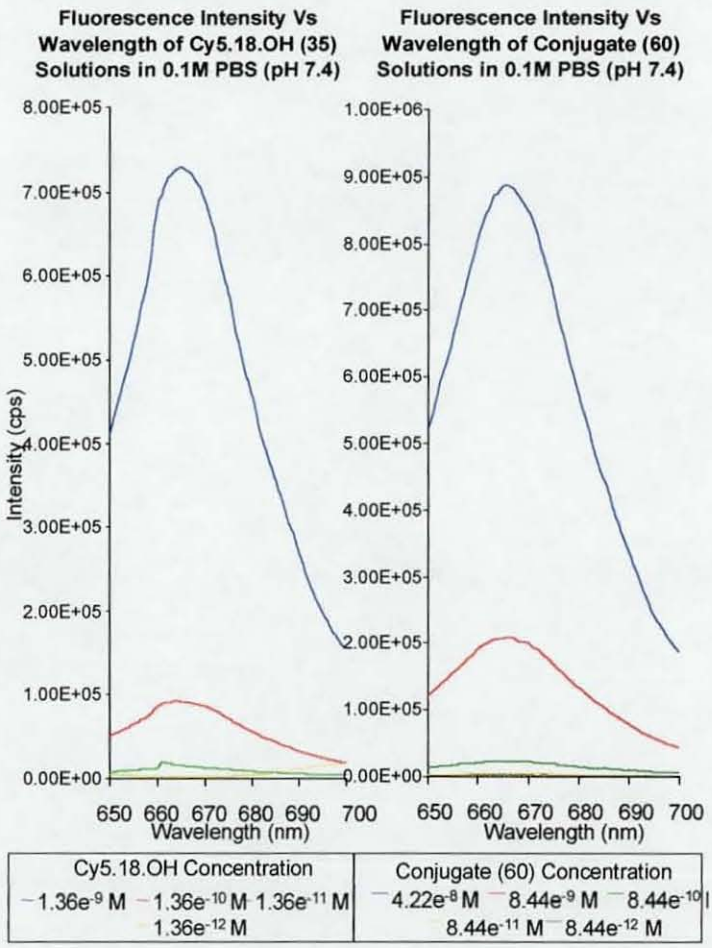
4.10.1.2 Fluorescence Spectra of Conjugate (60)

The fluorescence spectra of Cy5.18.OH **(35)** and conjugate **(60)** were obtained using the parameters in Table 4.19 and are displayed in Figure 4.26.

Table 4.19 Fluorimeter Parameters Used to Generate Emission Spectra of Cy5.18.OH (35) and Conjugate (60) in Solution

Excitation Wavelengths		**Emission Wavelength**		660 – 750 nm	
Cy5.18.OH **(35)**	650.1 nm	**Increment**	1 nm	**Integration**	0.5 s
Conjugate **(60)**	650.7 nm	**Slits**		1.25 mm all around	

Figure 4. 26 Comparison of the Emission Spectra of Cy5.18.OH (35) and Conjugate (60) in 0.1 M PBS Solution



Comparing these spectra it was possible to estimate the relative quantum efficiency of the conjugate, as previously described in Section 4.7.3. The

Cy5.18.OH fluorophore **(35)** gave I_f = 7.29e^5 cps at concentration of 1.36e^{-9} M, with Φ = 0.28, ε = 200,000 $M^{-1}cm^{-1}$.[161] Conjugate **(60)** gave I_f = 8.86e^5 cps at 4.22e^{-8} M, ε = 89,300 $M^{-1}cm^{-1}$, therefore Φ was calculated as 0.31. Waggoner *et al.* reported that the quantum efficiency of Cy5.18.OH **(35)** is higher in organic solvents than aqueous solution and the quantum efficiencies of its derivatives, especially Cy5.18.OH labelled amines, depend strongly on solvent viscosity, for example, the conjugates are 10 times more fluorescent in glycerol than water.[104]

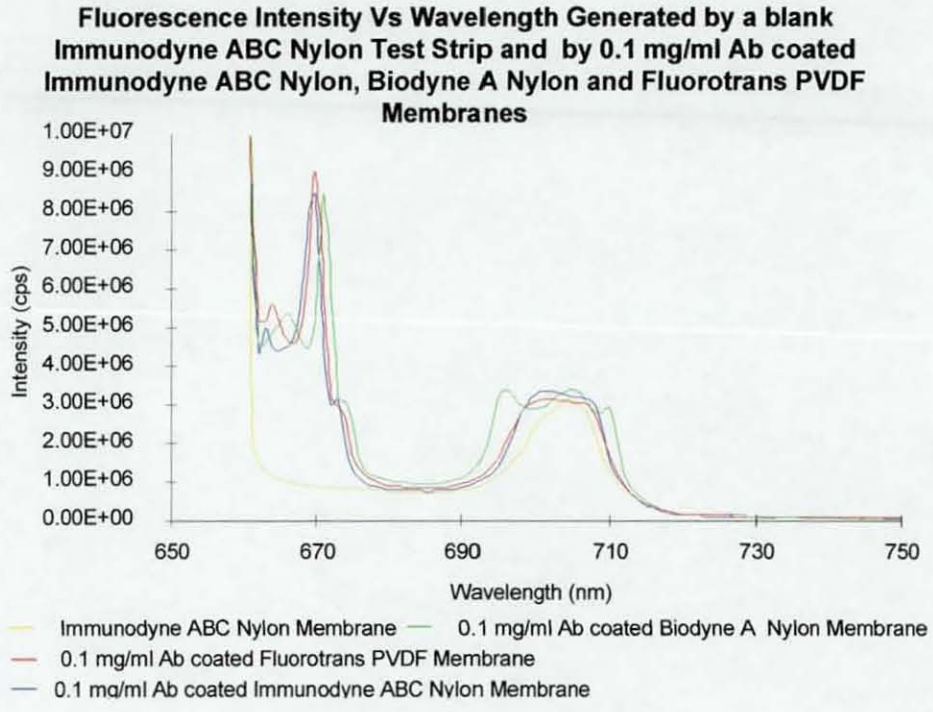
4.10.1 Autofluorescence of Membranes at 650 nm

Immunodyne ABC, Biodyne-A and Fluorotrans PVDF membranes were tested for matrix autofluorescence in the 650 – 750 nm range, as outlined in Section 4.7.1. The fluorescence of the test strips were determined by running an emission spectrum with the parameters given in Table 4.20 and the spectra are displayed in Figure 4.27 as a plot of Fluorescence Intensity (cps) Vs Wavelength (nm).

Table 4.20 Fluorimeter Parameters Used to Detect Matrix Autofluorescence

Excitation	650 nm	**Emission**	660 – 750 nm	**Increment**	1 nm
Integration	1 s	**Slits – Excitation**	2.5, 2.5 mm (9.0, 9.0 nm)		
		Emission	1.25, 1.25 mm (4.5, 4.5 nm)		

Figure 4.27 Emission Spectra of 0.1 mg/ml Antibody Coated Polymer Membrane Test Strips



On excitation at 650 nm a peak at 690 - 710 nm with Immunodyne ABC nylon was observed, which was due to Raman scattering, no other peaks were seen. On antibody coating peaks at 670 nm were seen that were possibly due to the antibody coating the strip, as it was known that components of serum can exhibit autofluorescence in the 600 nm region.[175] It was discovered that on the antibody coated strips, the peak at 690 - 710 nm were also due to talc from the powered gloves worn whilst performing this experiment.

4.10.2 Detection of Sulfamethazine using Conjugate (60)

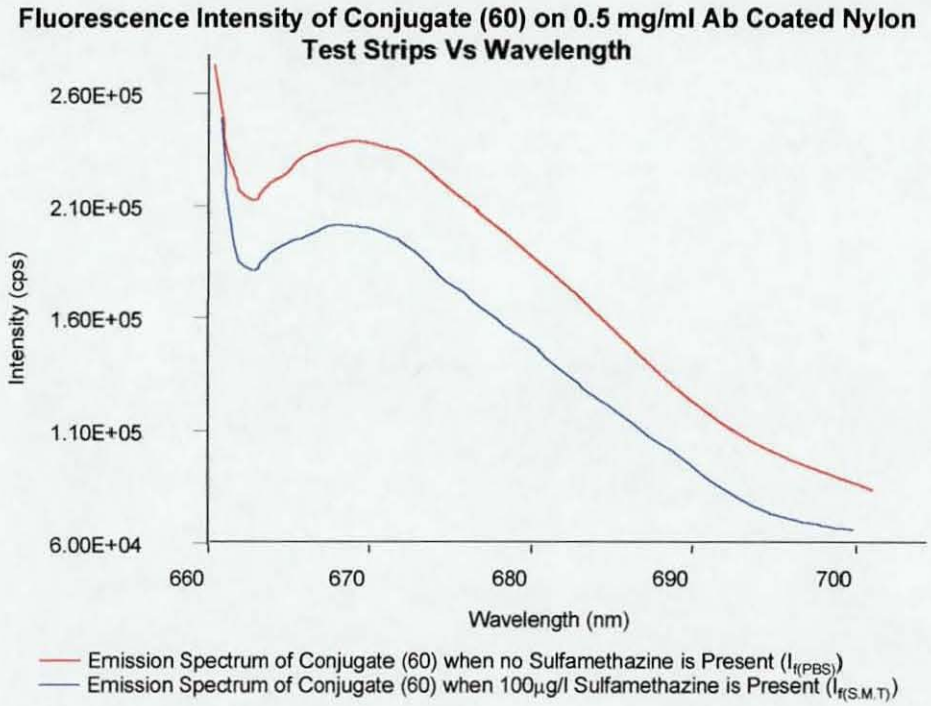
It was important to determine if sulfamethazine can be detected with conjugate **(60)**. It was therefore decided to run an assay on the strips using the conjugate and test strips (0.5 mg/ml Ab) prepared as described in Section 4.9.1. These were incubated in PBS or sulfamethazine (100 μl, 100 μg/l) then conjugate **(60)** (100 μl, $8.30e^{-7}$ M) as described in Section 4.9.2. The fluorescence emission intensities were determined using the standard parameters given in Table 4.21 below, and the spectra are

displayed in Figure 4.28 as Fluorescence Intensity (cps) Vs Wavelength (nm).

Table 4.21 Standard Parameters Used to Generate an Emission Spectrum for Conjugate (60)

Excitation	650 nm	**Emission**	660 – 700 nm	**Increment**	1 nm
Integration	1 s	**Slits – Excitation**	2.5, 2.5 mm (9.0, 9.0 nm)		
		Emission	1.25, 1.25 mm (4.5, 4.5 nm)		

Figure 4. 28 Emission Spectra Produced on Competition of Conjugate (60) With Sulfamethazine on Nylon Test Strips



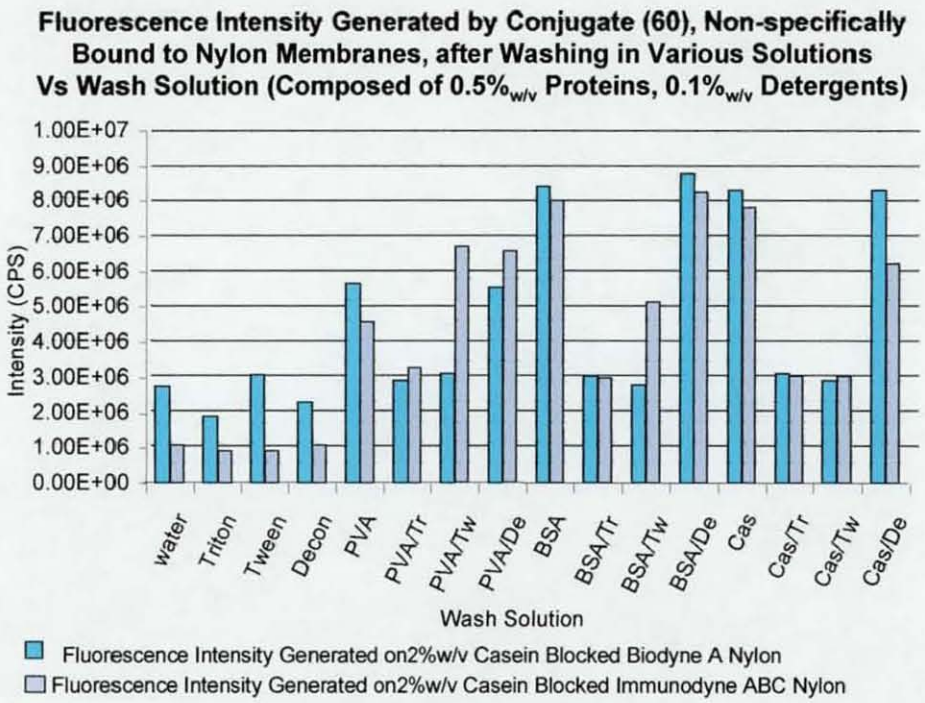
The fluorescence intensity generated by conjugate **(60)** decreased when sulfamethazine was present compared with the negative solutions. The maximum fluorescence intensities seen at 670 nm were lower than expected for this conjugate concentration and as a result, we optimised the washing conditions for the test strips using a more concentrated conjugate solution and less immobilised antibody.

4.10.3 Optimisation of Immunoassay Wash Conditions

The ability of the conjugate to non-specifically bind to Immunodyne ABC (0.45 μm pore nylon) and Biodyne-A (0.45 μm pore nylon) were compared in the presence of various combinations of polymers, proteins and detergent as a washing solution. The detergents used are outlined in the chequerboard displayed as in Section 4.8.4, Table 4.16.

Immunodyne ABC and Biodyne-A nylon strips containing no antibody, were prepared as described in Section 4.9.1. The strips were incubated in conjugate **(60)**, (100 μl, $1.66e^{-6}$ M), for 5 minutes and washed for 15 minutes using the polymer ($0.5\%_{w/v}$):protein ($0.5\%_{w/v}$):detergent ($0.1\%_{w/v}$) wash conditions outlined in Table 4.16. The emission spectra intensities were determined using the standard parameters (Table 4.21) and displayed in Figure 4.29 as a plot of Fluorescence Intensity (cps) Vs Wash solutions.

Figure 4.29 Comparison of Wash Conditions for Conjugate (60) on Immunodyne ABC and Biodyne-A Nylon Test Strip



It was observed that water or detergent solutions gave the best overall washing conditions. This was thought to be due to the characteristics of the conjugate **(60)**, which was made from fluorophore Cy5.18.OH **(35)** that

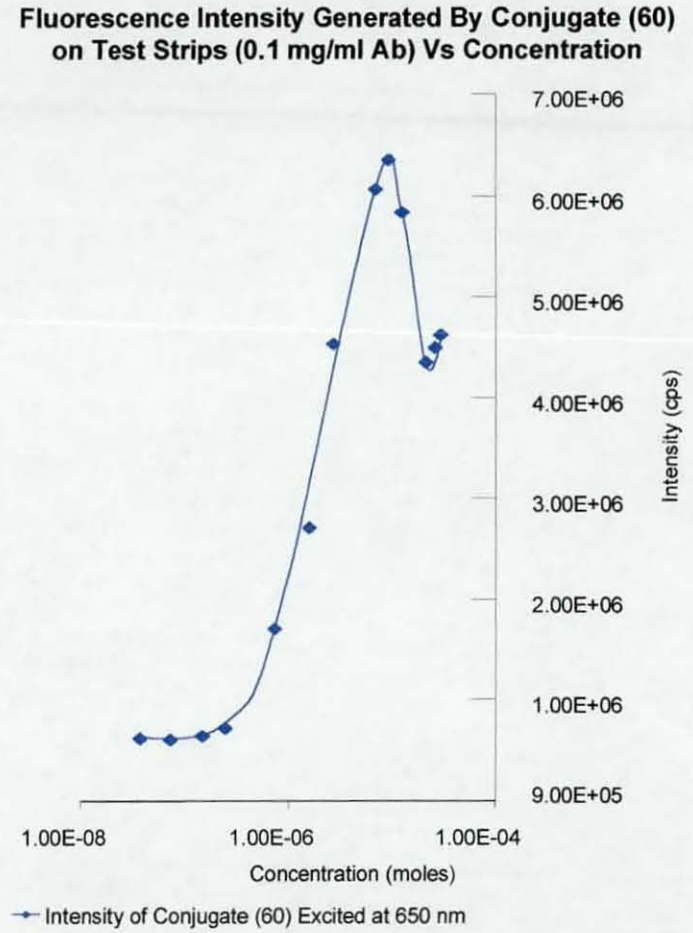
was designed for use in DNA labelling in aqueous conditions and had sulfonate groups incorporated into the structure to aid water solubility. These sulfonate groups make the conjugate **(60)** hydrophilic, which aids its removal from the membrane surface. The other wash solutions, containing polymers and proteins are less polar and were more suitable for conjugate **(52)**.

Both of the membranes are made of nylon 6,6, but the Biodyne-A is cast onto a non-woven polyester matrix and is inherently hydrophilic.[180] This was thought to be the cause of Biodyne-A giving higher intensities of fluorescence indicating higher non-specific binding to the membrane surface. It was decided to continue with the Immunodyne ABC nylon membrane and to wash with deionised water.

4.10.4 Optimisation of the Concentration of Conjugate (60)

The concentration of conjugate to be used in the assay was optimised on 0.1 mg/ml antibody coated test strips prepared as described in Section 4.9.1. The test strips were incubated for 5 minutes in the conjugate solution **(60)**, (100 μl, $7.5e^{-5}$ M – $7.5e^{-8}$ M), washed in deionised water (2 ml, 15 minutes) with orbital shaking, and blotted on a tissue. The fluorescence intensity was determined using the standard parameters outlined in Table 4.21 and expressed in Figure 4.30 as a plot of Intensity (cps) Vs Concentration (moles).

Figure 4.30 Concentration Curve of Conjugate (60) on 0.1 mg/ml Antibody Coated Test Strips



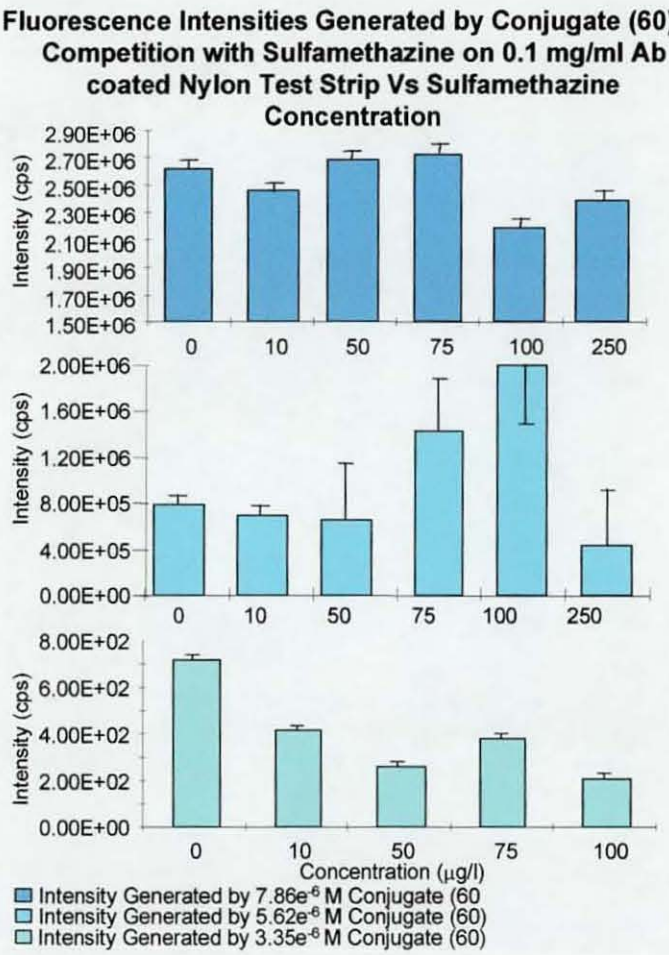
As the concentration of the conjugate increased the fluorescence intensity increased giving the same trend as seen with conjugate **(52)**. The fluorescence intensity reached a peak when $1e^{-5}$ M conjugate **(60)** was present in the solution. As the conjugate concentration increased beyond $1e^{-5}$ M, the observed intensity decreased as expected. This was due to fluorescence quenching, which was consistent with the argument raised in Section 4 8.3 for the fluorescein conjugate **(52)**.

4.10.5 Competitive Assay of Sulfamethazine with Conjugate (60)

The semi-competitive immunoassay procedure defined in Section 4.9.2 was used to test if sulfamethazine could be detected on a solid surface by conjugate **(60)**. The assay was performed using 0.10 mg/ml antibody coated strips prepared as outlined in Section 4.9.1 and a series of

sulfamethazine standards of 250, 100, 75, 50, 10 and 0 μg/l prepared as described in Section 2.3.5. The test strips were incubated for 5 minutes in the sulfamethazine standards (100 μl), washed in water for 1 minute with shaking, blotted and then incubated with the conjugate **(60)** (100 μl, $7.86e^{-6}$ M) for 5 minutes. The strips were washed in deionised water (2 ml, 15 minutes) with orbital shaking, blotted on a tissue and the fluorescence emission intensity was determined using the standard parameters (Table 4.21). The results are displayed in Figure 4.31 as Fluorescence Intensity (cps) Vs Sulfamethazine Concentration (μg/l).

Figure 4. 31 Competition Assay of Conjugate (60) and Sulfamethazine on 0.1 mg/ml antibody Coated Test Strips



Using $7.86e^{-6}$ M conjugate **(60)**, the observed intensity fluctuated significantly, and the expected decrease in fluorescence intensity with increasing sulfamethazine concentration was not observed. It was thought

that this was due to the conjugate concentration being too close to the maximum limit, which could cause no clear differences in fluorescence intensities to be seen. Therefore, the assay was repeated using a lower conjugate **(60)** concentration ($5.62e^{-6}$ M and $3.35e^{-6}$ M). The results are displayed in Figure 4.31 as a plot of Fluorescence Intensity (cps) Vs Sulfamethazine Concentration (μg/l).

With $5.62e^{-6}$ M conjugate an insignificant decrease in the intensity was seen with the addition of sulfamethazine until 75 μg/l was present, which gave a large increase in intensity. This could be due to interference from the test strip autofluorescence seen in Figure 4.27, light scattering reflecting off the white membrane surface in this wavelength region, or insufficient non-specific binding occurring during conjugate incubation. The latter argument could account for the rise in fluorescence seen at 75 μg/l, because the concentration of sulfamethazine non-specifically adsorbed on the membrane surface may by sufficiently high to affect the polarity of the membrane and aid conjugate binding. Alternatively, the antibody concentration used to detect the sulfamethazine is too high. With 0 – 50 μg/l sulfamethazine, two conjugate molecules are binding per antibody, which reduces the observed fluorescence due to quenching caused by fluorophore overcrowding. At 75 μg/l sulfamethazine, the majority of the antibodies contain sulfamethazine and a conjugate **(60)** molecule, the excess antibodies having two conjugates and exhibiting a reduced fluorescence as stated before. At 100 μg/l a 1 : 1 ratio of conjugate and sulfamethazine is found on the antibody, and at 250 μg/l competition between the conjugate and sulfamethazine is occurring. Therefore, the antibody concentration needs to be reduced to 0.001 mg/ml.

The $3.35e^{-6}$ M gave an overall downward trend but the observed intensity was very small making this conjugate concentration unworkable because insufficient fluorescence counts are accumulated to ensure that the results are statistically significant.

To increase the conjugate working range it was necessary to decrease the antibody and increase the conjugate concentrations on the test strip. The 0.1 mg/ml antibody concentration was suitable for conjugate **(52)** but

this was unsuitable when using conjugate **(60)**. Therefore the antibody concentration for this conjugate as well as assay incubation times, washing times and instrumentation needed to be optimised before a working assay could be achieved.

We decided to stop the immunoassay development with the Cy5.18.OH **(35)** conjugates and concentrate on producing a longer wavelength conjugate, which had a λ_{max} compatible with the commercially available laser diodes (750 – 800 nm), thus, the remaining research was focused on the production of a symmetrical heptamethine cyanine dye conjugate.

4.11 Longer Wavelength Dyes

The near infrared spectral region, 700 – 1200 nm, is an area of low interference, where only a few classes of molecules exhibit significant absorption or fluorescence. The combination of the limited endogenous fluorophores along with the much lower interferences from Rayleigh and Raman scattering ensures reduced background fluorescence. This reduced background allows excellent limits of detection to be achieved, even with fluorophores whose absorptivity and fluorescence quantum efficiencies are only moderate.[126]

4.11.1.1 Heptamethine Cyanine Dye Conjugates

The symmetrical heptamethine cyanine dyes **(36)** and **(51)** used to label sulfamethazine in this study have λ_{max} of 765.0 and 782.0 nm and ε of 250,000 and 135,000 $M^{-1}cm^{-1}$, respectively. [104,154]

The first heptamethine cyanine dye conjugate **(57)** was made using the Cy7.18.OSucc **(48)**, activated with EDC, the sulfamethazine-linker compound **(38)** was added and an amide produced. The Cy7.18.OSucc **(48)** starting material was photo-instable and, although the reaction was performed in the dark, the dye had started to decompose as the conjugation reaction was taking place. This made the purification of the conjugate very difficult.[104] However, a small sample of conjugate was

isolated and tested for antibody specificity. The ELISA assay gave a $B/B_{0(50\%)}$ of $3.6e^{-6}$ M that was 360 times less active than sulfamethazine.

It was noted that on formation of the conjugate **(57)** the λ_{max} was reduced to 745 nm from 765 nm of the parent dye, Cy7.18.OH **(36)**, when recorded in PBS. The emission spectrum of Cy7.18.OH **(36)** gave a maximum at 788 nm in PBS, whereas the conjugate **(57)** gave an emission maximum at 777 nm. Therefore the Stokes shifts changed from 23 nm for Cy7.18.OH to 32 nm on conjugation. Nevertheless, due to the reduced antibody specificity no further investigations were performed on this conjugate.

The synthesis of heptamethine cyanine dye conjugate **(61)** was based on fluorophore **(51)**. This fluorophore was symmetrical and has a stabilising cyclohexene ring situated at the central position of the methine bridge.[182] The approach taken to create conjugate **(61)** was the incorporation of sulfamethazine into the structure of the fluorophore itself, unlike the other conjugates where the sulfamethazine was labelled after fluorophore synthesis.

The sulfamethazine was introduced into the fluorophore starting materials by attacking 2-chloro-3-{(E)-1-hydroxymethylidene]-1-cyclohexene-1-carbaldehyde **(49)** with succinic anhydride in the presence of butyllithium. The product of this reaction was coupled to sulfamethazine using EDC and this sulfamethazine containing starting material was used to synthesise the fluorophore in the standard way (Section 3.4.10).[156] The purification of this compound was easier than that of conjugate **(57)** as the product was more photostable. The green compound produced had λ_{max} = 782 nm, ε = 61,000 $M^{-1}cm^{-1}$, and a Stokes shift of 28 nm. The antibody specificity checked by ELISA gave a $B/B_{0(50\%)}$ of $1.1e^{-6}$ M which was 11 times less active than the sulfamethazine standards. Unfortunately, the ^{1}H-NMR data was not consistent with the dye **(51)** or as expected for the conjugate **(61)**, thus, due to the lack of structural information no further immunoassay investigations were performed on this conjugate.

CHAPTER FIVE

CONCLUSIONS AND FURTHER WORK

5.1.1 Conclusions

Several heterogeneous competitive immunoassays were developed, which by definition, used limited amounts of antibody and conjugate and employed reagent separation steps.

The beginning of the research concentrated on the synthesis of the enzyme labelled sulfamethazine conjugates **(37A)** and **(37B)**, to be used as tracers in an ELISA assay. The HRP-sulfamethazine conjugate **(37B)** was used to develop the first test strip immunoassay, using 0.45 mg/ml antibodies immobilised on a Millipore NC matrix; the free-sites on the membrane were blocked using $1\%_{w/v}$ PVA and a TMB chromogenic substrate was the colour-generating agent. Conjugate **(37B)** and sulfamethazine **(1)** directly competed for the antibody and a satisfactory response was seen within 17 minutes. The HRP assay had the advantage of enzyme amplification and used the rapidly reacting TMB reagent to give the assay.

At this point a limited amount of work was done on the method of sulfamethazine extraction, it was found that the ultrasonication of a meat sample would give a satisfactory extraction of sulfamethazine, this was tested by ELISA and by the test strips.

The fluoroimmunoassay development started when the fluorescein labelled sulfamethazine conjugates **(52)** and **(53)** were synthesised by direct and indirect labelling, respectively. The directly labelled sulfamethazine conjugate **(52)** had an excitation maximum at 493 nm, an emission maximum at 525 nm, ε of 32,000 $M^{-1}cm^{-1}$ and was used in further immunoassay developments. The fluoroimmunoassay did not have the advantages of signal amplification that was experienced with HRP, so the assay was designed to give a decrease in fluorescence signal with increasing sulfamethazine concentration.

The study of the immunoassay optimisation essentially relied on the combination of the physico-chemical model, describing the binding reactions between the antibody, sulfamethazine and conjugate **(52)**; and

the reduction of background interference from non-specific binding. On optimisation of the immunoassay conditions with conjugate **(52)** we found that a 0.1 mg/ml antibody coated Immunodyne ABC nylon test strip (where the free-sites of the membrane were blocked with a $2\%_{w/v}$ casein solution) gave the required sensitivity. The washing conditions were optimised and a 15 minute wash in 0.5% BSA + 0.1% Triton x-100 in PBS wash solution was used. The ideal concentration of conjugate was found to be $1e^{-5}$ M this gave single fluorophore occupancy per antibody and avoided the fluorescence quenching problems that occurred when two conjugate molecules were bound. Complications in the immunoassay arose when powered latex gloved were used by the operator, as the talcum powder from these gloves was fluorescent and when excited at 493 nm and gave several emission peaks between 510 – 535 nm which interfered with the fluorescein signal. Problems due to the transfer of sulfamethazine to the conjugate incubation solution were also detected as these gave rise to erroneous results. The inclusion of a second wash step removed this problem and had the advantage of improving the assay precision (%CV = 1.25).

Optimisation of the immunoassay conditions resulted in a qualitative test strip with a sensitivity of 10 μg/l and a 26 minutes assay time, which was suitable for sulfamethazine screening. The reduction of the assay time to 10 minutes gave a less sensitive assay than the 26 minute test, but the sensitivity was still below the 100 μg/l required by the European Union.

The aim of the project was to develop a near infrared fluoroimmunoassay, consequently the far-visible/near-infrared dye Cy5.18.OH **(35)** was synthesised and reacted with sulfamethazine to give conjugate **(60)**. Conjugate **(60)** had an excitation maximum at 650 nm, an emission maximum at 670 nm and ε of 89,000 $M^{-1}cm^{-1}$.

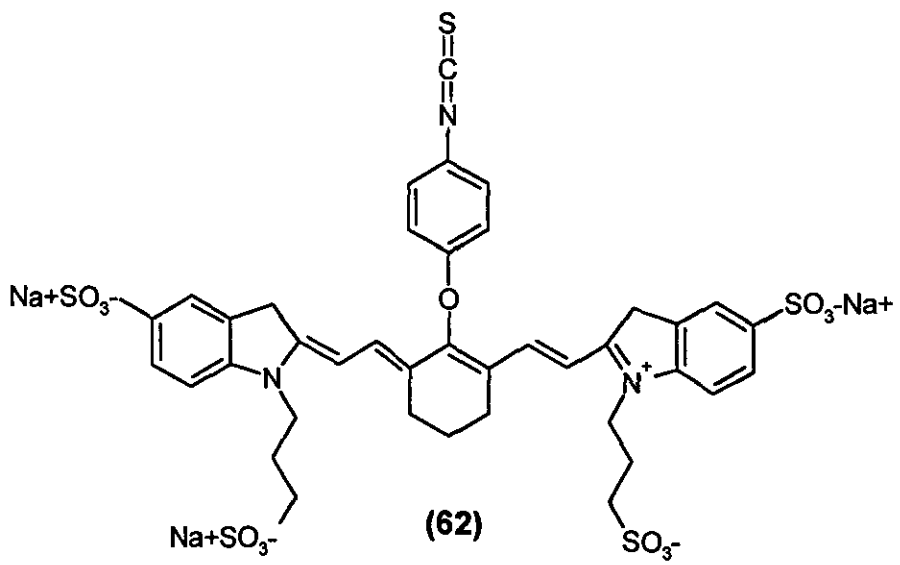
When tested on a 0.5 mg/ml antibody coated Immunodyne ABC nylon test strip, the pentamethine cyanine dye conjugate **(60)** showed a difference between a sulfamethazine positive and negative sample. This encouraging result lead to the optimisation of the washing conditions and conjugate concentration. At this point, it became obvious that the antibody concentration being used was too high. As conjugate **(60)** was in limited

supply we decided not to use this to optimise the immunoassay conditions, but to focus on the synthesis of heptamethine cyanine dye conjugates that had excitation wavelengths greater than 750 nm range as these would be more compatible with commercially available laser diodes. Unfortunately, the attempted syntheses of these longer wavelength fluorophores were not successful.

5.1.2 Further Work.

The logical extension of this research covers several areas of interest, namely: the development of a sensitive generic test that can detect all sulfonamides, a test strip that does not contain any proteins or biological reagents, and the incorporation of an internal reference/control system. This would be followed by the automation of the assay procedures and the development of a hand-held detection system, which would enable point of activity testing for sulfonamides in food testing laboratories, supermarkets, abattoirs, dairies and farms.

The approaches adopted would include the development of a heptamethine cyanine conjugate of sulfamethazine to be used as a generic tracer. Recently a new dye (NN382) **(62)** has become commercially available.



(62)

This fluorophore contains an isothiocyanate group in the centre of the ring stabilised heptamethine bridge that can be conjugated to sulfamethazine giving a thiourea linkage. This dye has a λ_{max} of 810 nm that is compatible with inexpensive laser diodes. Narayanan has suggested that the conjugation should take place in a sodium carbonate/saline buffer (pH 10.5, 0.5 M CO_3^{2-}, 1 M NaCl) in a 1:1 ratio and stirred in the dark for 3 hours. Separation is by gradient HPLC using a tetraethylammonium acetate buffer.

The removal of polyclonal antibodies from the system would improve the test strip sensitivity, would overcome the problem fluorescence quenching on the divalent antibodies, and would improve the shelf-life of the test strip. Ideally we would use a stable polymer membrane that irreversibly binds to the sulfonamide group with equal affinities for all the sulfonamides, and does not disrupt the fluorescence of the tracer. It has been proposed that a polymer matrix that includes anthracene residues could be used for this purpose. Once the anthracene polymer was proven to be suitable for sulfonamide binding, then an internal reference or control could be developed, for example, detecting the change in fluorescence of the polymer as an indication of antigen binding. Alternatively, the test could incorporate an electrochemical detection that was controlled by the insertion of the test strip into the instrument. This could detect a potential that is specific to the polymer-conjugate complex, or the detection of antibodies from the sample binding to the matrix surface.

A final goal would be the development of a hand-held instrument that has a detection limit comparable with a standard bench top model and can be used for point of activity testing.

APPENDICES

APPENDIX 1

Table A 1 Maximum Residue Limits of Antibiotic and Antimicrobial Compounds

Substance	MRL	Part Of Animal
Chloramphenicol	10 μg/kg	All edible tissues
Sulfonamides	100 μg/kg	All edible tissues
Trimethoprim	50 μg/kg	All edible tissues
Nitrofurans	5 μg/kg	All edible tissue
Dapsone	25 μg/kg	All edible tissue
Dimetridazole	10 μg/kg	All edible tissue
Ronidazole	2 μg/kg	All edible tissue
Febantel	1 mg/kg, 10 μg/kg	Liver, All other tissue
Fenbendazole	1 mg/kg, 10 μg/kg	Liver, All other tissue
Oxfendazole	1 mg/kg	Liver
Ivermectin	15 μg/kg, 20 μg/kg	Liver, All other tissue
Levamisole	10 μg/kg	All edible tissue
Carazolol	50 μg/kg	Liver, kidney
Azaperone	100 μg/kg, 50 μg/kg	Kidney, All other tissue
Benzylpenicillin Ampicillin Amoxycillin	50 μg/kg	All edible tissue
Oxacillin Cloxacillin	300 μg/kg	All edible tissue
Tetracycline	600 μg/kg	Kidney
Oxytetracycline	300 μg/kg	Liver
Chlortetracycline	100 μg/kg	All edible tissue
Clenbuterol	0.5 μg/kg	All edible tissue

as Stated by the EC Regulation 92/675 and EEC 2377/90

APPENDIX 2

Table A 2 Chemicals and Reagent Grades

Chemical	Grade	Chemical	Grade
3-Methyl-2-butanone	98%	Toluene	-
1 –Bromododecane	97%	Tween-80	-
1 –Butanol	99.8%, HPLC	Phosphorus oxychloride	98%
1,12-Diaminododecane	98%	12-Bromo-1-dodecanol	99.9%
1,2-Propanediol	99+%	1,4-Butanesultone	AnalaR
‡‡1,3,3-Trimethoxypropene	96%	1,2-Diaminethane	98%
1,8-Diaminooctane	98%	Polyethylene glycol 20,000	-
1-Propanol	Absolute	2,3,3-Trimethylindolenine	97%
3,3',5,5'- tetramethyl benzadine	99%	1,2-Dichlorobenzene	99%, anhydrous
4-Chloro-1-naphthol	99%	2-Propanol	AnalaR
§§4-hydrazino benzenesulfonic acid	99%	N,N,N'N'- tetramethyl ethylenediamine	AnalaR
6-Bromohexanoic acid	98+%	Cyclohexanone	99.8%
Acetic acid	99.8%	Hydrochloric acid	37%w/v
Acetic anhydride	99+%	N-Bromosuccinimide	99%
Benzene	99+%, HPLC	Acetone	99%, AnalaR
Carmazine Red	-	‡‡Albumin (bovine)	Fraction V
Chloroform	99.8%	Boric acid	Lab grade
D_4-Methanol	NMR grade	Chaps	-
d_6-DMSO	NMR grade	Citric acid	Anhydrous
Decon-90		d-Chloroform	NMR grade
Dichloromethane	HPLC	Deuterium oxide	NMR grade
Disodium hydrogen	AnalaR	DBU	-

‡‡ Avocado (Lancashire, UK)

§§ Lancaster (Lancashire, UK)

phosphate			
DMF	Anhydrous	Diethyl ether	99%, AnalaR
DMF	HiPerSolv	Sodium hydroxide	97%
§§§EDC	-	Ethanol	Absolute
Fluorescein isothiocyanate	90%, Isomer 1	Ethyl acetate	AnalaR
Hexamethyl phosphoroustriamide	AnalaR	Fluorescamine	-
Hydrogen peroxide	35%$_{w/v}$	Gluconaldehyde dianil. Hydrochloride	99%
IgG (sheep serum)	Reagent	****Horseradish peroxidase	44,000
Lysine	-	N-Hydroxysuccinimide	98%
N,N'-Disuccinimidyl carbonate	Tech	Iodoethane	99%
Napthol Blue Black	-	Methanol	99.8%, AnalaR
n-Butyllithium	1.6M	Disodiumtetraborate	AnalaR
p-Benzoquinone	-	Ammonium sulfate	AnalaR
Polyvinyl alcohol 10K	99+%	††††2-Bromo-1,1,3-trimethoxypropane	90%
Potassium acetate	99.7%, AnalaR	Casein	Tech
Sodium azide	99.99+%	Pyridine	99.5%, AnalaR
Sodium carbonate	AnalaR	Potassium hydroxide	98%
Sodium dihydrogen phosphate	AnalaR	Sodium borohydride	GPR
Sodium nitrite	AnalaR	Sodium chloride	AnalaR
Sodium periodate	-	Sodium hydrogen carbonate	AnalaR
Succinic anhydride	99+%	Sodium perborate	
Sucrose	-	Sodium sulfate	AnalaR

§§§ Pierce and Warriner (Cheshire, UK)

**** Biozyme (Gwent, UK)

†††† Fisher Scientific (Loughborough, UK)

THF	AnalaR	N,N'-Succinimidyl carbonate	AnalaR
TMS	NMR grade	Sulfamethazine	AnalaR
Triethylamine	AnalaR	Tetraethylammonium acetate	Tech
Tween-20	-	Triton X-100	-
Triethylamine hydrochloride	98%		

APPENDIX 3

A 3.1 Synthetic Experimental Data

Figure A.3. 1 Raman Spectrum of Water

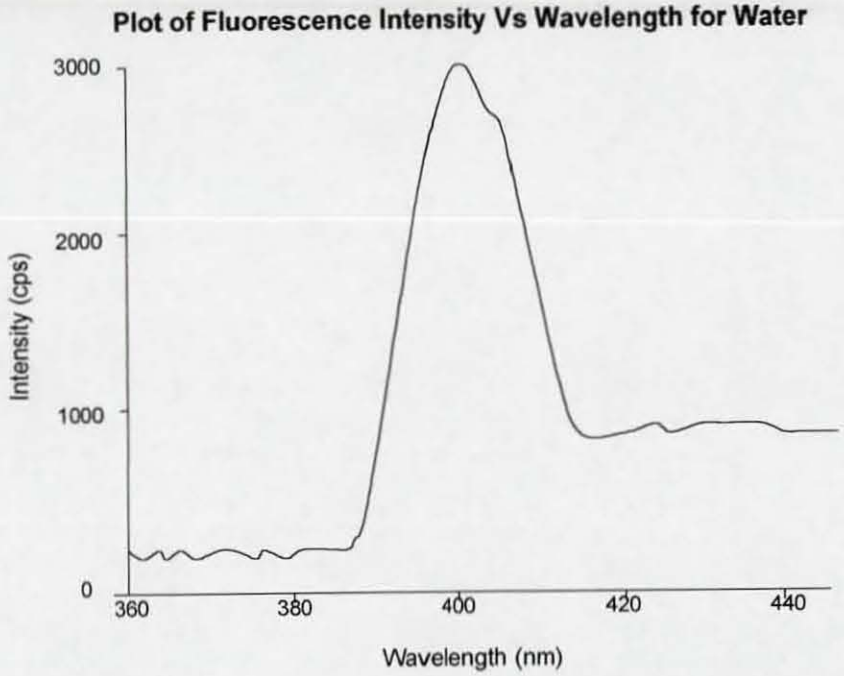


Figure A.3. 2 Binding Efficiencies of Conjugates (52) and (60)

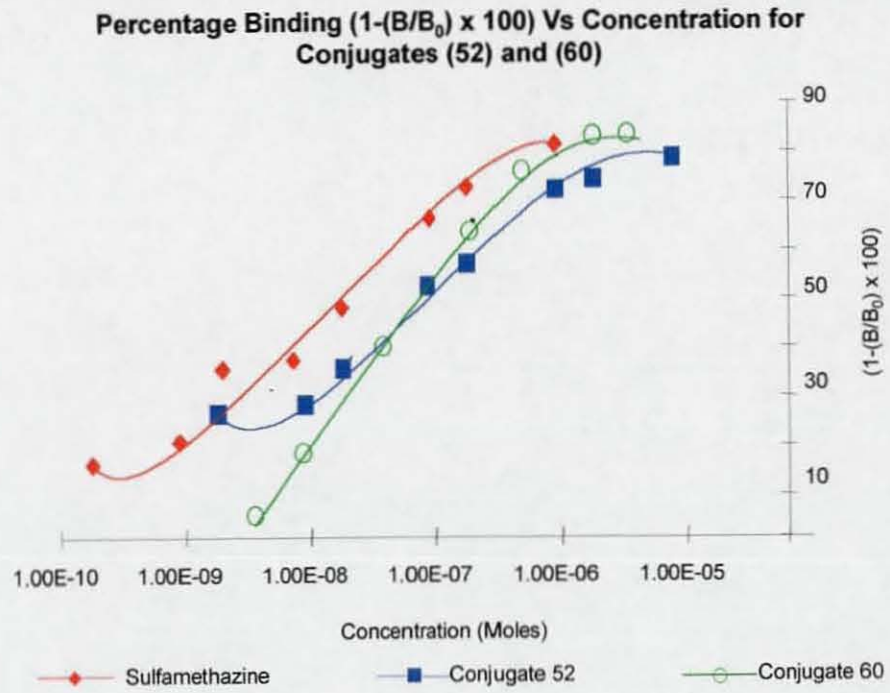


Figure A.3. 3 Plot of ELISA Assay Results for Conjugate (54)

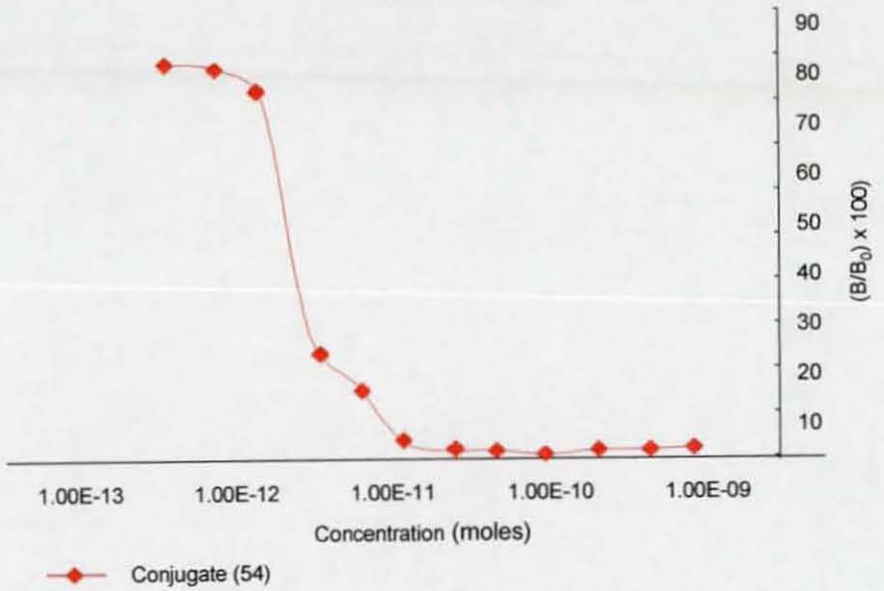


Figure A.3. 4 Binding Efficiency of Conjugates (55)

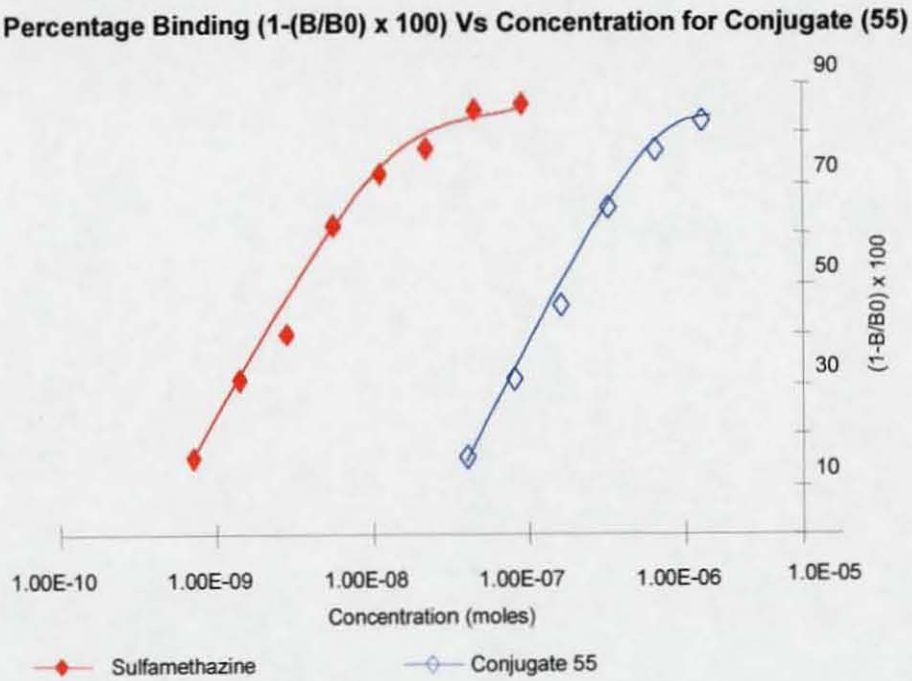


Figure A.3. 5 Binding Efficiencies of Conjugates (56) and (57)

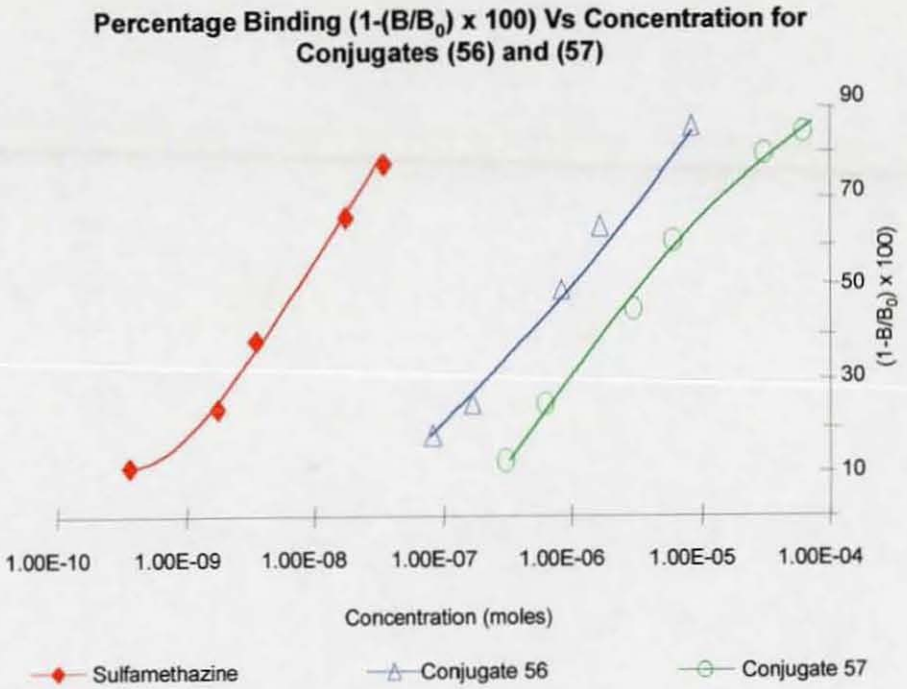


Figure A.3. 6 Binding Efficiency of Conjugates (59)

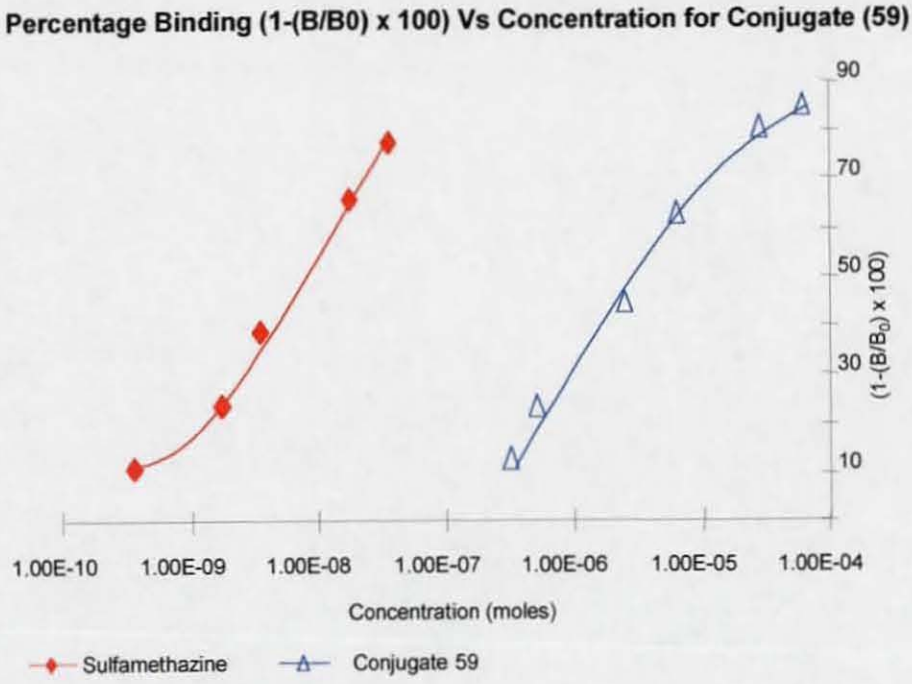
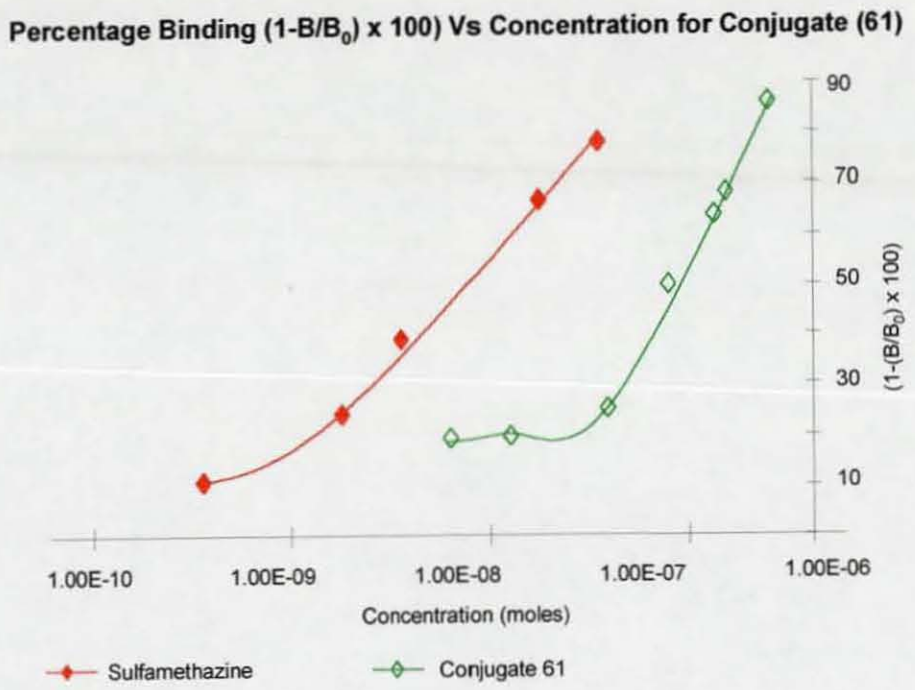


Figure A.3. 7 Binding Efficiency of Conjugates (61)



A 3.2 Molar Absorptivities of Sulfamethazine Conjugates

Figure A.3. 8 Molar Absorptivity of Fluorophore (51)

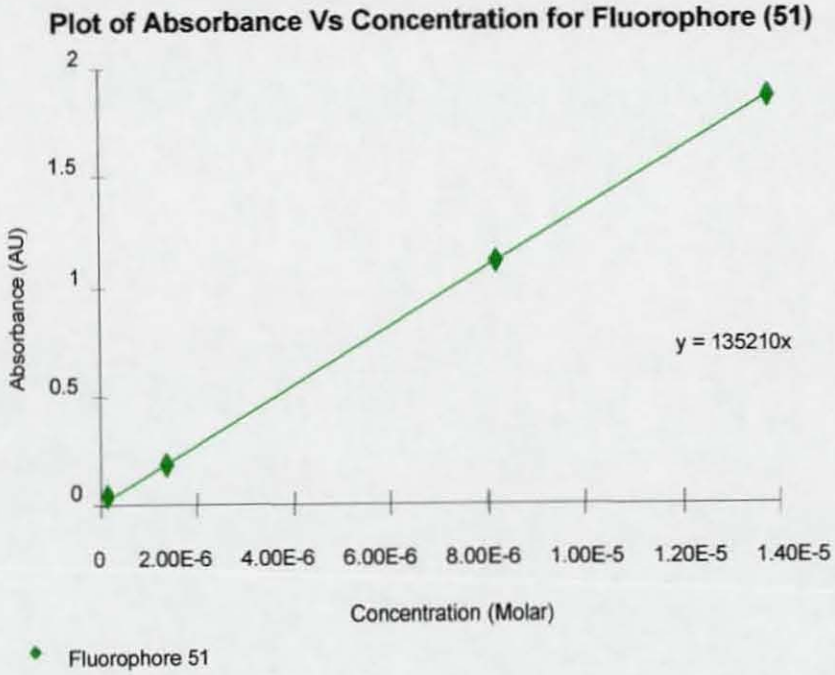


Figure A.3. 9 Molar Absorptivity of Fluorophore (55)

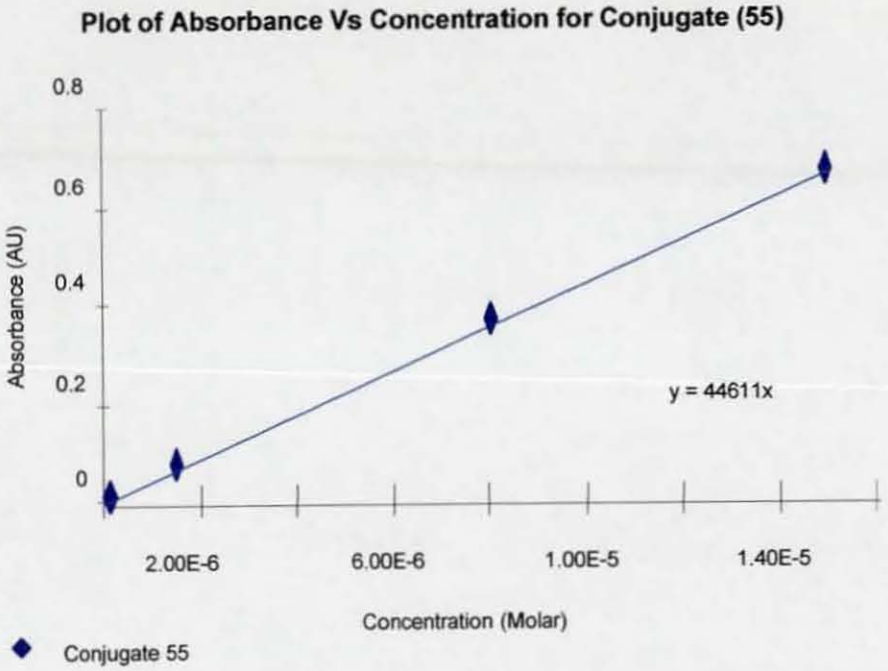


Figure A.3. 10 Molar Absorptivity of Fluorophore (56)

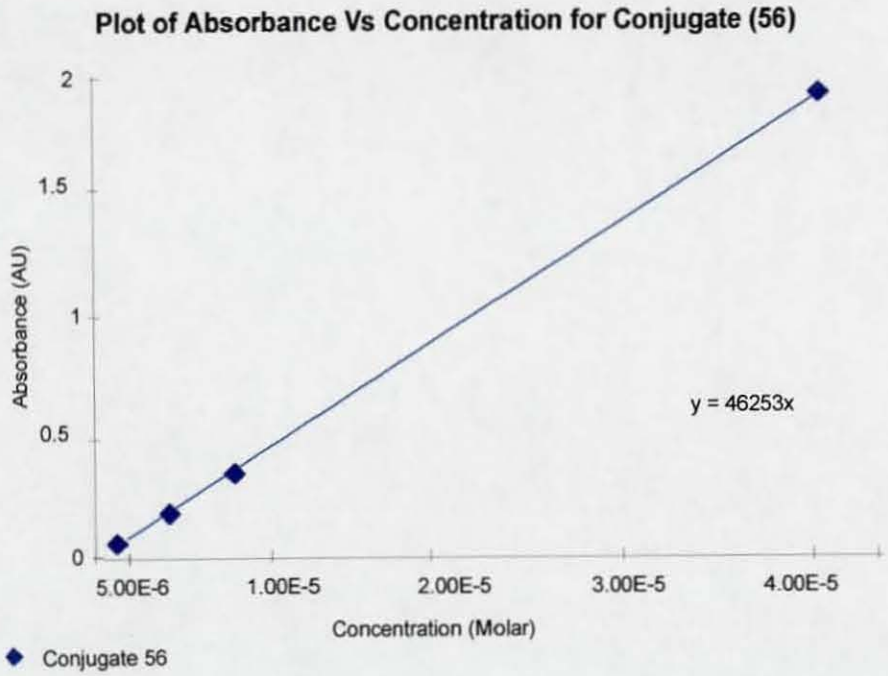


Figure A.3. 11 Molar Absorptivity of Fluorophore (60)

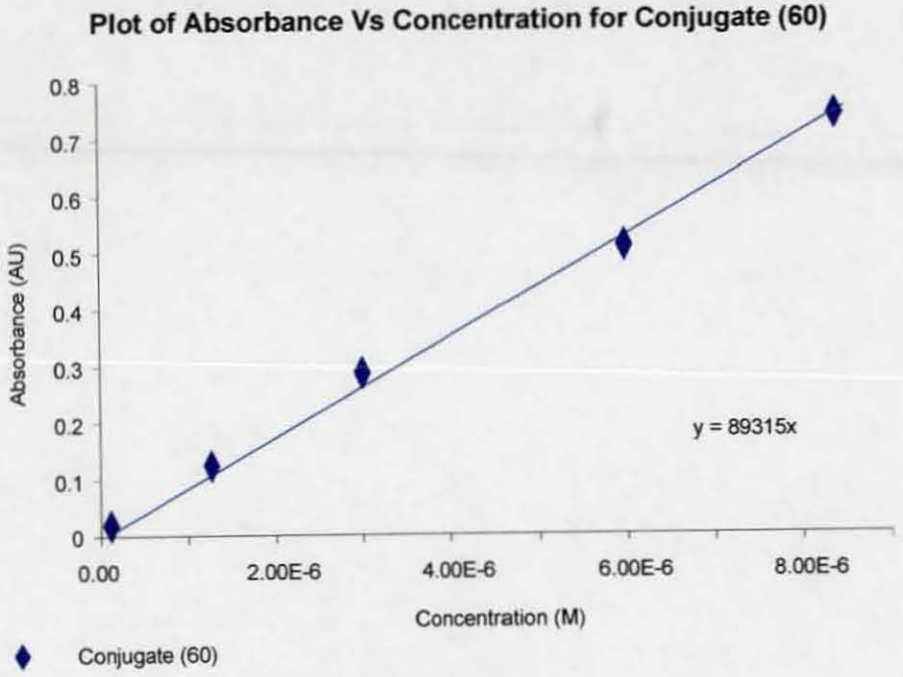
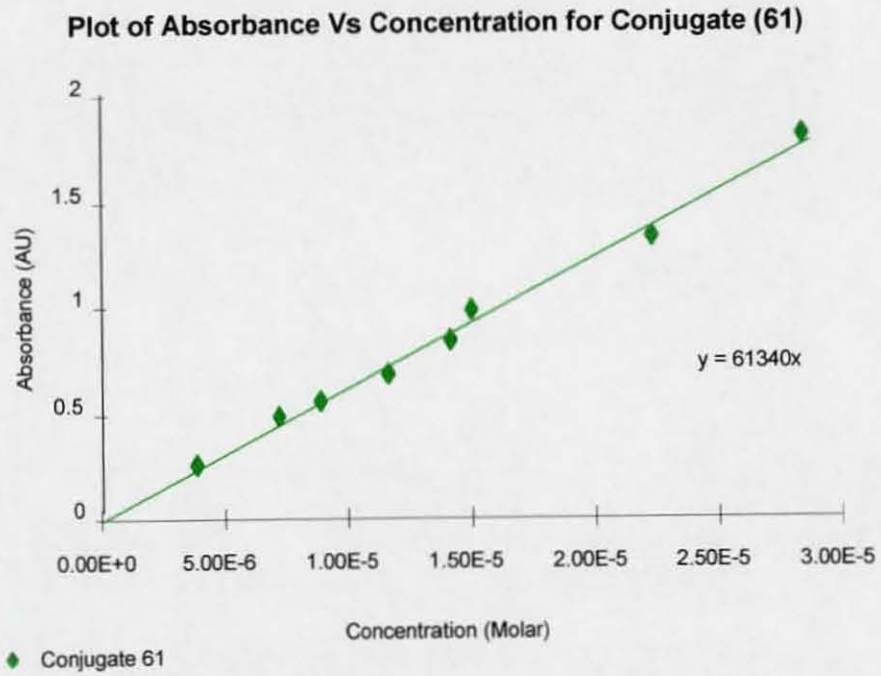


Figure A.3. 12 Molar Absorptivity of Fluorophore (61)



APPENDIX 4

Table A 4.1 Membrane Sources and Selection

Membrane/Batch	Material	*Manufacturer*	Comment	Value
Biotrace NT 0.45μm	NC	Gelman	Clean spot	++++
Polymacron	Polymer	Kalyx	Total staining	+
NC - 0.45μm Mylar backed	NC	Millipore	Good	+++++
NC - 0.45μm Unbacked	NC	Millipore	Handling problem	++
NC - 0.45μm Mylar backed	NC	Millipore	Ring effect	++
NC - 3μm	NC	Millipore	Irregular antibody dispersion	+
Immobilon AV	PVDF	Millipore	Hydrophobic	+
Immunodyne ABC 0.45μm	Nylon	Pall	Good	+++++
Biodyne-A	Nylon	Pall	Good	++++
Immunodyne ABC 1.2μm	Nylon	Pall	Good antibody dispersion	+++
Fluorotrans	PVDF	Pall	Good	+++
PBNC 120/404	NC	Schleicher & Schuell	Irregular antibody dispersion	+++
Protran BA 85 4009/601	NC	Schleicher & Schuell	Irregular coating	++
Nytran12 00528/1	Nylon	Schleicher & Schuell	Irregular coating	++
Protran BA 85 4009/601	NC	Schleicher & Schuell	Not strong staining	++
Nytrans Plus 6251/602	Nylon	Schleicher & Schuell	Total staining	+
Protran BA 85 2022/506	NC	Schleicher & Schuell	Ring effect	+
220/2	PDVF	Schleicher & Schuell	Hydrophobic	+
125/405	NC	Schleicher &	Irregular	+

		Schuell	antibody dispersion	
18/407	NC	Schleicher & Schuell	Irregular antibody dispersion	+
Nytran13 00545/1	Nylon	Schleicher & Schuell	Ring effect, high background	+
Gerber 0.45μm	NC	Whatman	Slight ring	+++
NC - 0.45μm Unbacked	NC	Whatman	Too soft to handle	++
1872/74	NC	Whatman	Reject	+

Table A.4.2 Cross-Reactivities of Sulfamethazine Antisera[75]

	Cross reactivity (%) at concentration		
Drug	**100 μg/l**	**1000 μg/l**	**10000 μg/l**
Sulfamethazine	100	100	100
Sulfamerazine	75	31	20
Sulfadiazine	7.9	3.8	2.3
Sulfathiazole	0	1.5	0.5
Sulfaguanidine	4.3	0.3	0.04
Sulfaquinoxaline	22.3	5.0	1.1
Chloramphenicol	0	0	0
Penicillin G	0	0	0
Penicillin V	0	0	0
Ampicillin	0	0	0
Cloxacillin	0	0	0

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