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Optical Modulation of High-Affinity Biomolecules Function via Photochromic Dyes:

A Step towards an Artificial Control of Biological Activity

Cranfield Health

PhD Thesis

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Academic Year 2007 - 2008

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Optical Modulation of High-Affinity Biomolecules Function via Photochromic Dyes: A Step towards an Artificial Control of Biological Activity

Supervisor

Prof. David C. Cullen

Submitted September 2008

This thesis is submitted in partial fulfilment of the requirements of the degree of Doctor of Philosophy

Abstract

Prior to this study, there has yet to be a clear demonstration of an artificial control of antibody affinity via photochromic dyes. The research described in this thesis sets out to address this by investigating photochromic dyes and their subsequent applications with high affinity biomolecules - primarily to photomodulate the functions of biomolecules. The main avenue of investigation explored the conjugation of photochromic dyes (spiropyrans) to proteins (an enzyme and five different antibodies), to achieve reversible photomodulation of protein function for possible applications in biosensor technology (such as the development of reagentless bio-reversible sensing systems). A secondary aim involved the investigation of the feasibility of antibody-antigen binding in the presence of ionic liquids. Ionic liquids have recently experienced growing interest as replacements for traditional organic solvents in a number of industrial applications. The practicability of spiropyrans in ionic liquids was also investigated (with the future possibility of photomodulated antibody-antigen interactions in ionic liquids to deliver a variety of improved analytical performances).

The synthesis and photoswitching properties of an appropriate range of spiropyran dyes are reported. The spiropyran dyes are synthesised to possess a carboxyl group to aid carbodiimide mediated conjugation to lysine amino groups of proteins. The photochromic behaviour of the spiropyran dyes in various solvents, temperature and pH ranges were observed. Conjugation of carboxylated spiropyran dyes to an enzyme: horseradish peroxidise, was initially observed to aid development of experimental protocol for the target study group *i.e*. antibodies. Photomodulation of the modified horseradish peroxidase was found to demonstrate ~ 60 % decline in enzyme activity, an effect which was reversible as a result of the photoswitching capabilities of the attached spiropyran dyes. The five different antibodies; anti Atrazine, anti GroEL, anti Phytanic Acid, anti FITC and anti *Staphylococcus aureus* were modified with spiropyran dyes as with horseradish peroxidase. Reversible antibody affinity photomodulation was observed via their reaction in an ELISA which

yielded a decline of ~ 15 %, ~ 40 %, ~ 50 %, ~ 55 % and ~ 65 % in binding signal respectively. A fatigue assessment was conducted on the photoswitching capabilities of both the conjugated and the unconjugated spiropyran dyes. This was expressed as ten photoswitching cycle experiments, the first evenly spaced over ten days and a second over ten weeks. The initial results suggested dye degradation increased with consecutive photoswitching cycles of the conjugated spiropyran dyes. It was observed that the level of degradation for the unconjugated spiropyran dyes was independent to the timing interval between photoswitching cycles, suggesting storage stability of the compound. However the level of degradation for the conjugated spiropyran dyes was dependent to the timing interval between photoswitching cycles, suggesting storage instability.

A subsequent study involved the demonstration of the feasibility of antibody-antigen binding in ionic liquids for the first time. Various combination ratios of ionic liquids with aqueous phosphate buffered saline were employed. Initial experimentation of antibody-antigen binding showed that use of solutions with an ionic liquid content of 50 % and below, produced identical results to that of the standard aqueous phosphate buffered saline. At 95 % ionic liquid content, a lower level of binding activity was observed. The possibility of a photomodulated antibody-antigen interactions in ionic liquids did not produce a significant result on this occasion with the observation of spiropyran dyes failure to photoswitch in solutions with as low as 10 % ionic liquid content.

In summary, although the development of a reagentless bio-reversible sensing system continues beyond the period of this PhD thesis, significant progress has been made with regards to photochromic antibodies as possible candidates for further studies and applications, also the establishment of antibody-antigen binding in various ionic liquids can serve as ways to further enhance the applicability of such reactions under different environmental conditions.

Acknowledgements

This research has been fully funded by the Biotechnology and Biological Sciences Research Council (BBSRC) with support from the University of Leicester, UK (ScioniX Ltd) and Aberdeen University, UK.

I would like to express my gratitude to my supervisor Prof. David C. Cullen for the opportunity given (which included challenges afforded) in undertaking this exciting project. Many thanks to my associate supervisor Dr. Kal Karim, and Dr. Michael J. Whitcombe's help with the chemistry related aspect.

To all the staff, students and friends at Cranfield Health, it was a pleasure to know and work with you. I would like to give a special mention to Dr. Antonio Guerreiro, Dr. Paul Wilson and Miss Mariliza Derveni for their generosity of time and support.

To my family, thank you for your support in various ways. Rebecca for ensuring I am always fed. Benjamin, Dennis and David for encouraging me to "finish hard". Amanda and Elke for proof reading my work a hundred times.

Last but not the least, God Almighty for making it possible for me to get this far with regards to my education.

"Count your blessings, name them one by one,

And it will surprise you what the Lord has done"

Johnson Oatman, Jr.

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Abbreviations

Å	Ångstrom
Ab	Antibody
Abs	Absorption
Ag	Antigen
BSA	Bovine Serum Albumin
BSA-FITC	Bovine Serum Albumin Fluorescein Isothiocyanate
CAP	Catabolite Activator Protein
CE-TMI-I	L-(2-Carboxyethyl)-2,3,3-Trimethylindolenium Iodide
CTC	Cis, Trans, Cis
CTT	Cis, Trans, Trans
DCC	*N,N′*-Dicyclohexylcarbodiimide
DES	Deep Eutectic Solvents
DMAPN	Dimethylaminopropionitrile
EDC	1-(3-Dimethylaminopropyl)-3-Ethylcarbodiimide
ELISA	Enzyme-Linked Immunosorbent Assay
Fab	Fragment Antigen Binding Region of an Antibody
Fc	Fragment Crystallisable Region of an Antibody
FITC	Fluorescein Isothiocyanate
GFPdenat	Denatured Green Fluorescent Protein
GMP	Guanosine Monophosphate
HFP	Hexafluoro-2-Propanol
HRP	Horseradish Peroxidase
IC_{50}	Inhibitory Concentration 50%
IgG	Immunoglobulin G
IR	Infra Red
K_a	Affinity Constant
LEDs	Light Emitting Diodes
MC	Merocyanine Dye
MEK	Methyl Ethyl Ketone (2-Butanone)
MES	2-[N-Morpholino]Ethanesulfonic Acid

NHS	N-Hydroxy Succinimide
NMR	Nuclear Magnetic Resonance
OD	Optical Density
PAP	Phenylazophenylalanine
PBS	Phosphate Buffered Saline Solution
PBST	Phosphate Buffered Saline and Tween 20 Solution (0.5%)
P-EDC	Polymer Bound EDC (1-(3-Dimethylaminopropyl)-3-Ethylcarbodiimide)
PPD	Purified Protein Derivative
R_f	Retention Factor
scAb	Single Chain Antibody Fragment
scFv	Single Chain Fv Antibodies
SP	Spiropyran Dye
SP-A	Spiropyran Modified Anti Atrazine Fragment
SP-Ab	Spiropyran Modified Antibody
SP-COOH	Carboxylated Spiropyran Dye
SP-COOH-Br	Bromo Derivative of a Carboxylated Spiropyran Dye
SP-COOH-Me	Methoxy Derivative of a Carboxylated Spiropyran Dye
SP-F	Spiropyran Modified Anti Fluorescein Isothiocyanate
SP-G	Spiropyran Modified Anti GroEL
SP-HRP	Spiropyran Modified Horseradish Peroxidase
SP-P	Spiropyran Modified Anti Phytanic Acid
SP-S	Spiropyran Modified Anti Staphylococcus Aureus
SP-Sigma	Commercially Available Spiropyran Dye
SPR	Surface Plasmon Resonance
Sulfo-NHS	*N*-Hydroxysulfosuccinimide
TLC	Thin Layer Chromatography
TMB	3,3',5,5'–Tetramethylbenzidine
TTC	Trans, Trans, Cis
TTT	Trans, Trans, Trans
UV	Ultraviolet

Chapter 1

INTRODUCTION AND LITERATURE REVIEW

1 Introduction and Literature Review

1.1 Overview

The notion of synthetic molecular switching is not a novel concept. Terms such as *pH indicators* and *transitions lenses* are common to the non-scientific individual. pH indicators (such as litmus paper) are one of the oldest classical forms of synthetic molecular switches; they display distinct colours based on the pH environment they are subjected to. Plastic photochromic (transitions) lenses are an example of synthetic optical molecular switches. The lenses darken when exposed to ultraviolet (UV) rays from sunlight, and revert to normal when the UV rays are absent. Both of these concepts rely on the capability of action modulation by a stimulus; *i.e.* whether by chemicals or photons or other phenomena.

Photochromic materials have attracted much attention recently because of their potential applications as optical filters, optical switches, optical memories and other similar molecular devices (Grofcsik *et al.*, 2002). They are compounds that are able to exist in very distinctive isomerisable forms; they can change their form following illumination at different ranges of wavelength (*i.e.*, visible [700-400nm] and UV [>390-200 nm]). This unique property renders them a potential core element in synthetic optical molecular switching technology (Pieroni *et al.*, 2001). Thus, photochromic materials are promising materials for applications in devices in which photomodulated functions are appropriate.

The binding technique of antibody-antigen technology has found widespread acceptance in the biomedical, biochemical and biotechnological communities. Its use has extended from quantitative analysis of hormones, drugs, proteins and enzymes to applications in infectious disease, food industries, agricultural products, and environmental toxicology. This is primarily attributed to the high affinity binding constants and specificity of antibody-antigen interactions, and the simplicity of the analytical technologies involved. However, as with most technology, improvements are usually made based on the shortfalls encountered, one of these being regeneration of antibody for re-use. Antibodies themselves are expensive, and current regeneration

processes after antibody-antigen complex formation are known to have an adverse effect on the functions of the antibodies over time. This limits the number of successive uses of antibodies, making their application less cost effective (Kress-Rogers, 1996; van der Gaag *et al.*, 2003). In spite of the fact that various antibody regeneration processes have been employed or considered, the idea of a photomodulated antibody affinity control highlights an alternative approach.

Photochromic materials (molecules) conjugated to polypeptides have demonstrated their potential as "smart" biological materials that switch *on* and *off*. The photo-isomerisation effect (of the photochromic molecules) can induce conformational changes of the entire macromolecule; bringing about a reversible switching mechanism (Mecheri *et al.*, 2003). Thus, photomodulation of antibody affinity may significantly assist in improving the regeneration process, in essence decrease or eliminate some of the harsh mechanisms currently employed. The possibility of a non-damaging regeneration step of antibody-antigen technology may also advance the application of related technologies, *i.e.* light regulated molecular switches that reversibly control biomolecular function. This can provide new opportunities for controlling activity in diagnostics, affinity separations, bioprocessing, therapeutics, and bioelectronics applications (Shimoboji *et al.*, 2002).

Ionic liquids are salts with poorly coordinated ions, resulting in them being liquids at temperatures below 100° C (some even at room temperature). These solvents have a low melting point, effectively zero vapour pressure, amongst other properties (Yang *et al.*, 2005). The proposed application is to use ionic liquids as solvents with zero vapour pressure which will eliminate the instability caused by the rapid evaporation of traditional water based solvents, and possibly as solvents that will allow biomolecules to function in stable thin solvent films. An example of a potential benefit is enabling application of antibody-antigen technology under extreme environmental conditions such as outside the earth's atmosphere (due to the zero vapour pressure of ionic liquids). This application is focused on delivering a variety of improved analytical performance of the antibody-antigen technology, which can be further applied to a photomodulated antibody-antigen interaction.

1.1.1 Structure of Thesis

The body of work presented in this thesis begins with an introduction to photochromism, photochromic dyes and photomodulation, and its existence in nature. With a varying scope of published work exploiting photomodulation; a selection of such previous investigations is presented. These range from *in vitro* applications of photomodulation in biological systems, to its application in non biological systems. The synthesis and characterisation of a specific group of photochromic dyes involved in this study is then presented. This also includes investigations on the properties and conditions for photoswitching.

The photomodulation experimental sections are divided into two main parts. The first section involves the development of protocol to photomodulate a protein (horseradish peroxidase) activity in a predominantly aqueous environment. The second focuses on the establishment of photochromic antibodies under the same environmental conditions, monitoring the modulation of antibody affinity by light.

The concluding secondary part involves an introductory environmental investigation on antibody-antigen binding activity in ionic liquids (possible alternatives to traditional solvents) and subsequently the potential in photochromic antibodies activity in ionic liquids.

1.2 Introduction to Photomodulation

1.2.1 Photochromism and Photochromic Dyes

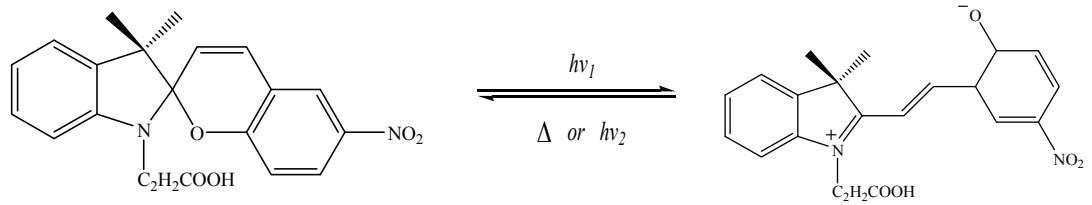
Photochromism can simply be defined as a light-induced reversible change of colour; however Bouas-Laurent *et al.*, (2001) gave a more precise definition:

> "Photochromism is a reversible transformation of a chemical species induced in one or both directions by absorption of electromagnetic radiation between two forms, A and B, having different absorption spectra. The thermodynamically stable form A is transformed by irradiation into form B."

The "chromic" phenomenon can be induced by various external stimuli. As also explained by Bouas-Laurent *et al.*, (2001), multi-mode chromism can occur in complex systems triggered alternatively by two or more different external stimuli, such as light, pH, temperature, salts, etc. Photochromism as the name suggest is triggered by light (photons). Thermochromism is triggered by the change in environmental temperature. Solvatochromism depends on the polarity of the solvent present. Electrochromism is induced by the gain and loss of electrons. Some of these effects will be briefly revisited in later chapters.

Photochromic dyes are substances that undergo reversible colour changes when exposed to light of an appropriate wavelength. These dyes can undergo structural changes and as such display changes in their absorption spectra. The dyes revert to their original form or colour when placed in the dark or upon irradiation of an appropriate wavelength. This reversible change in structure of the dye is known as photoswitching (Pieroni *et al.*, 2001). The photochromic behaviour is due to the ability of these compounds to exist in two different states, with relative concentrations dependent on the wavelength of the incident light. Examples of some photochromic dyes are illustrated in Figure 1.1.

Spiropyrans



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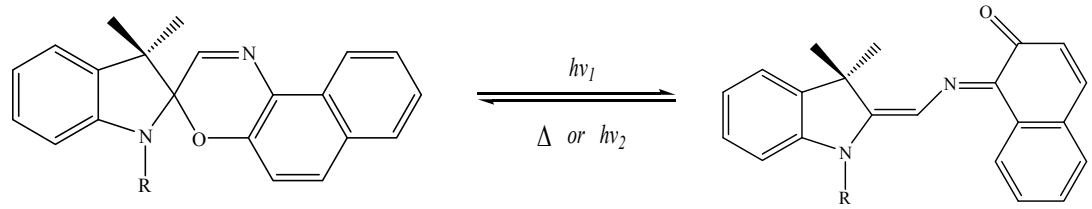
Spirooxazines



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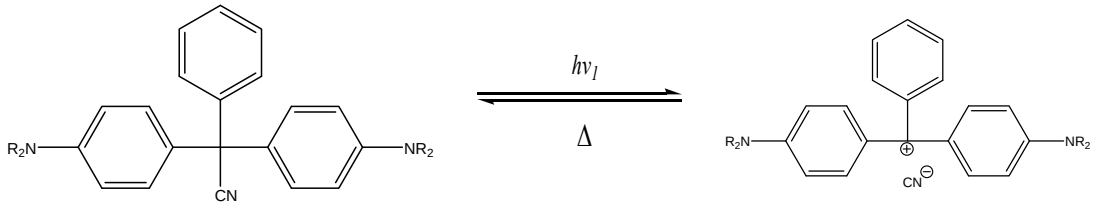
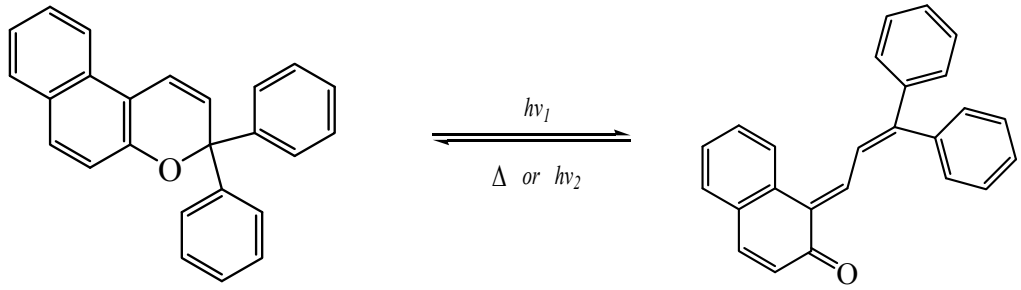
Triarylmethanes



Chromenes



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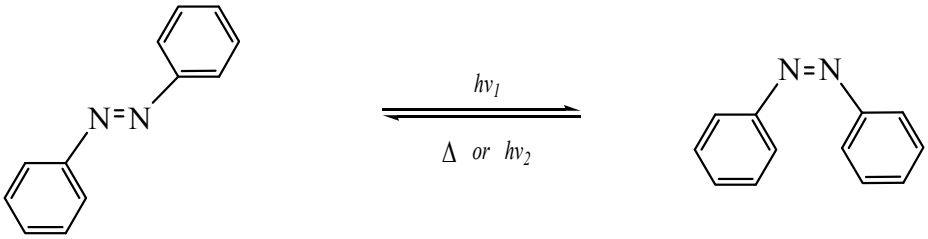
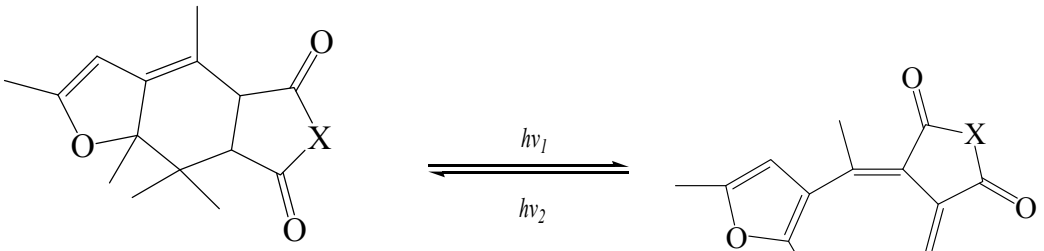
Azobenzene



Trans form Cis form

Fulgides and Fulgimides



X = O (fulgides)

X = NR (fulgimides)

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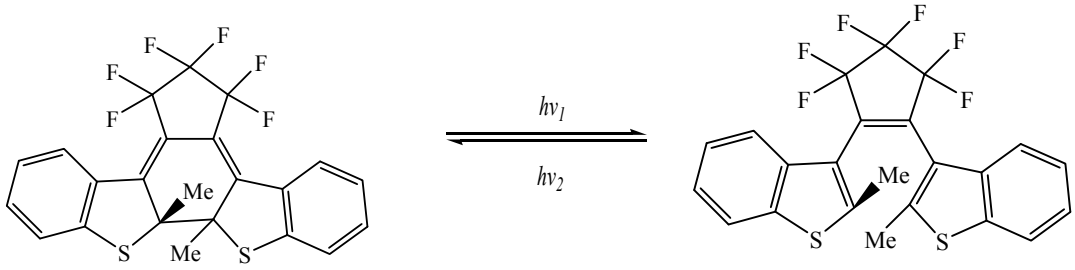
Diarylethenes and related compounds



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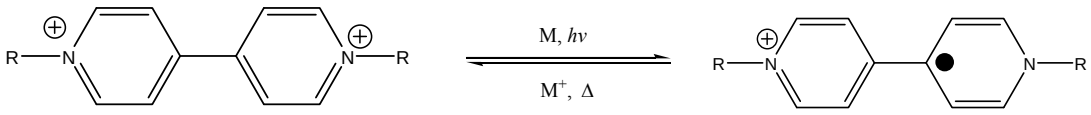
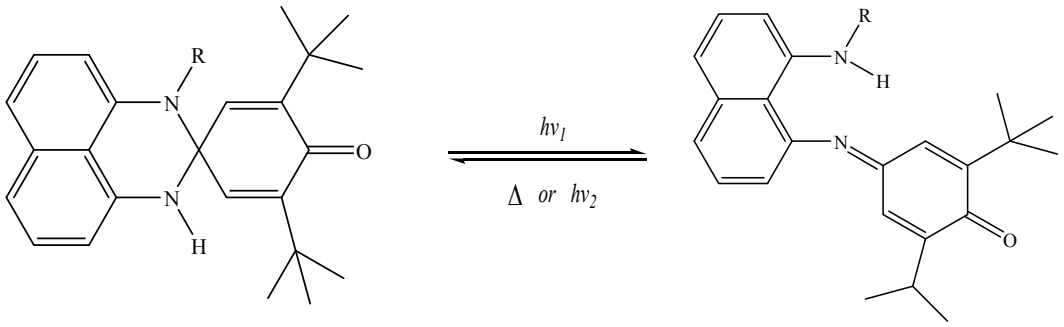
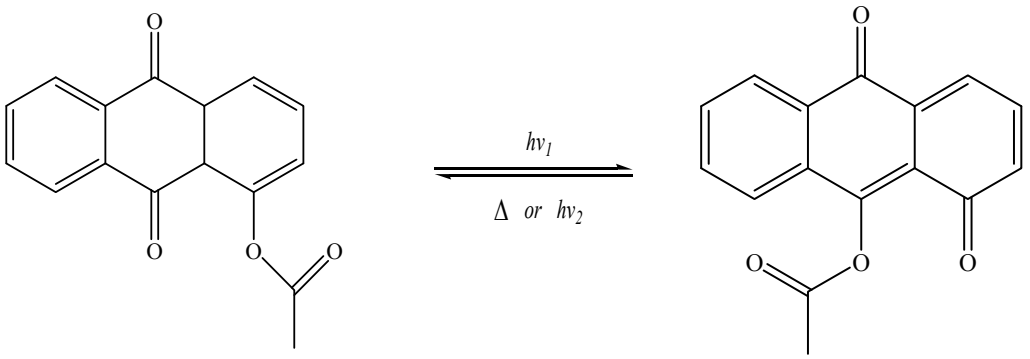
Viologens



Perimidinespirocyclohexadienones



Polycyclic quinones (periaryloxyquinones)



Spirodihydroindolizines

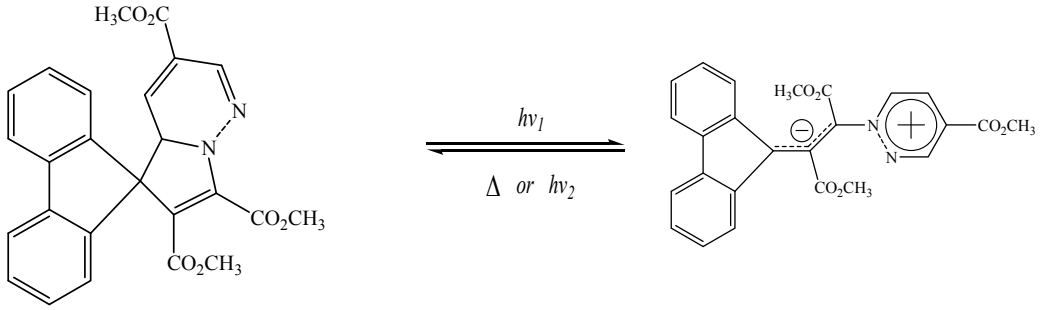


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Figure 1.1 : Examples of Photochromic Dyes with their Corresponding Isomers.

In these examples the thermally stable isomer is on the left, hence hv_2 denotes the longer illumination wavelength, usually between 700-400 nm, and hv_1 the shorter wavelength between 390-200 nm. However the wavelengths at which isomerisation occur depends on the particular structure of the dye and the environmental condition of the compound. Some of these dyes can also undergo multi-chromism denoted by Δ, a change in stimulus other than illumination. With regards to the violgens example, M denotes metals and ● denotes a free radical.

Photochromic dyes can be further described (based on colour change) as positive or negative photochromic systems. When the less thermodynamically stable form of the dye is deeper coloured, the system is referred to as "positive" (or normal) photochromic system; otherwise it is acknowledged as a "negative" (or inverse) photochromic system (Zhou *et al.*, 1995). These acknowledgements can however be ambiguous (exampled with Spiropyrans: when structurally modified with a carboxyl group; change from being a positive photochromic system to negative photochromic system). This observation further confirms the uniqueness of photochromic compounds.

Applications of photochromic dyes include: use as filters, in displays, in eye-protective laser goggles, in transition lenses and in self-developing photography. More recently these molecules have been applied in the photomodulation of enzyme activity; fluid flow visualisation; reversible holographic systems; as chromophores in 3-D optical data storage and as a means to photocontrol ion permeation through biological membranes (Khairutdinov *et al.*, 2001; Willner 1997; Berkovic *et al.*, 2000; Kawata *et al.*, 2000; Schomburg *et al.*, 2001).

General applications of photochromism can be divided into two categories (Bouas-Laurent *et al.*, 2001):

a) Those directly related to the change in absorption or emission spectra such as variable transmission optical materials, optical information storage, cosmetics and authentication systems.

b) Those related to other changes in physical or chemical properties such as refractive index, dielectric constant, electric conductivity, phase transitions, solubility, viscosity, surface wettability and secondary effects on biomolecules.

The principal motivation for the continued consideration of photochromic dyes for potential practical applications is the dye's ability to reversibly change structural forms. The use of photochromic dyes associated with proteins to modulate protein function has been limited.

1.2.2 Naturally Occurring Examples of Photomodulation

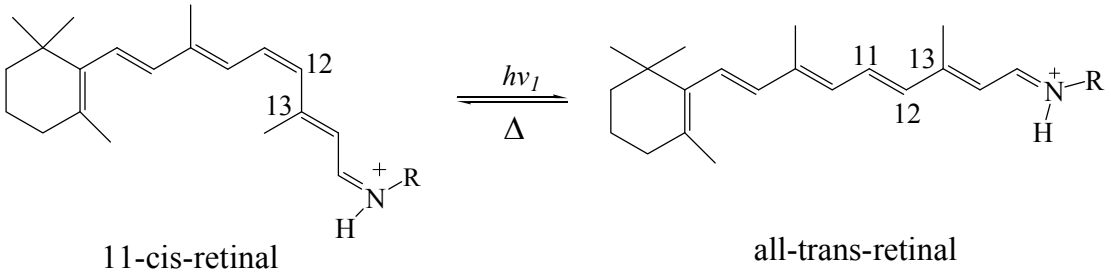
Photomodulation is simply a process of manipulating or regulating activity or function using optical wavelengths *i.e.* light source. Light is essential to life and most organisms are known to respond to it. Animals, plants and microorganisms benefit from the perception of light as a key to their environment (Hug *et al.*, 1991).

In nature, photochromic molecules represent the basic molecular triggers for many important biological photoreceptors, which recognise the intensity (quantity) and the wavelength (quality) of light in the environment. Many biological systems are photochromic, but few retain their photochromic properties when isolated from the living cell of which they are part. Photo-chemical and photo-physical reactions are fundamental to the interaction between light and organisms, triggering responses to light conditions through the modulation. In animals, light is a medium for conveying information on position, movement, shape and colour. In plants light is not only an information medium, but also used in photosynthesis and therefore of interest in itself; plants therefore obtain information about the amount, main direction and spectral composition of the light available (Hug *et al.*, 1991).

The incorporation of chromophores into proteins has been inherent in nature as far back as early primitive plant species; consequent alterations and developments have led to systems such as photosynthesis, photoreceptors and ultimately vision (Renner *et al.*, 2006). Among these naturally occurring biological proteins that undergo photomodulation (with absorption spectra within the visible light range of the electromagnetic spectrum), the more extensively studied biomolecules include retinal proteins (from rhodopsins in higher organisms to sensory rhodopsins in halobacteria), open-chain-tetrapyrrole proteins, and the xanthopsins found in eubacteria such as *Ecothiorhodospira halophila* (Ipe *et al.,* 2003).

Naturally occurring photomodulation such as rhodopsin is highlighted in Figure 1.2. Rhodopsin is a protein in the membrane of the rod photoreceptor cell in the retina of the eye. It contains a protonated retinal-Schiff's base complex which naturally lies in

the intermembrane pocket formed by seven trans-membrane α-helical receptors. The chromophore is bound to a protein via a lysine through the protonated Schiff base. Upon light detection rhodopsin undergoes a photochromic change from 11-*cis* to all-*trans* retinal (Figure 1.2).



11-cis-retinal all-trans-retinal

Figure 1.2 : The Photochromic Cycle of Rhodopsin.

The photochromic change from 11-*cis* to all-*trans* retinal. When the molecule is exposed to high intensity of light of a high wavelength ($h\nu_1$), the equilibrium shift favours the conversion to all-trans retinal. The reverse reaction takes place in the dark and is slow.

Photo-excited rhodopsin triggers an enzymatic cascade process resulting in the hydrolysis of Guanosine monophosphate (GMP). This in turn closes cation-specific channels within the rod cell membrane, which are naturally open to allow influx of Na^+ in the dark. The resulting hyperpolarisation causes the inner synaptic body to send a nerve impulse to other neurons in the retina aiding vision. Conversely there is a light-induced lowering of calcium ion levels within the nerve which aids recovery/ repolarisation of the excited neurons back to a passive "dark" state; the cycle begins again upon detection of light. It has also been observed that when rats are moved from a dark to a light environment, dopaminergic neurons are stimulated leading to activation of tyrosine hydroxylase resulting in enhanced dopamine synthesis. It is the light exposure which increases the formation of dopamine metabolites (Hug *et al.*, 1991).

Another example can be observed in plants. Phytochrome (P) controls the photomorphogenesis of plants. Chemically, phytochrome is comprised of a chromophore consisting of an open chain of four pyrrole rings bonded to the protein moiety which can exist in two conformations know as P_r and P_{fr}.

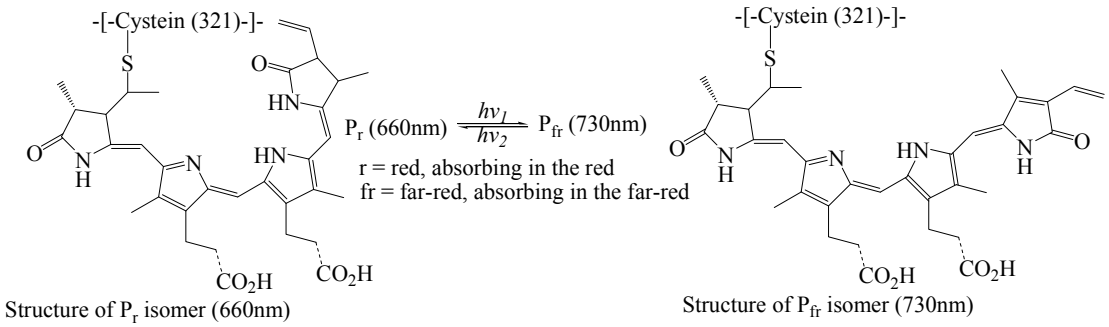


Figure 1.3 : The Photochromic change Phytochrome from P_r to P_{fr}.

The change in absorbance is caused by the photochromic change of phytochrome from P_r to P_{fr}. The red-absorbing form changes to the far-red absorbing form when it absorbs red light (660 nm) and back again when it absorb far-red light (730 nm).

P_{fr} is formed by irradiation of phytochrome with red light. Since daylight contains large amount of red light, during the day phytochrome is mostly converted to P_{fr}. At night, phytochrome will slowly convert back to the P_r form (Figure 1.3). Many flowering plants utilise this process to regulate the time of flowering based on the length of day and night (photoperiodism) and also to set circadian rhythms (roughly-24-hour cycle behavioral processes of living beings). It also regulates other responses including the germination of seeds; elongation of seedlings; the size, shape and number of leaves; the synthesis of chlorophyll and the straightening of the epicotyl or hypocotyl hook of dicot seedlings (Bouas-Laurent *et al.*, 2001; Hug *et al.*, 1991).

The general mechanism of action of naturally occurring photomodulation is characterised by the following salient common features (Ipe *et al.,* 2003; Pieroni *et al.*, 2001):

(a) They contain a photochromic molecule attached to a macromolecular protein matrix.

(b) On irradiation, the photochromic moiety undergoes reversible stereochemical rearrangements between two or more isomeric forms, the reaction direction being determined by the wavelength of the incident light.

(c) This primary photo chemical reaction induces a conformational change in the attached protein matrix, the "photosignaling state", which finally leads to the physiological response.

1.2.3 Designing Reversible Protein Photoswitches

The ability to duplicate the naturally occurring examples of photomodulation has been of interest for decades. This involves the artificial design of proteins linked to photochromic dyes (photoswitches), whose activity can be reversibly photomodulated as biological systems with a switch. Over the past 3 decades, a limited number of artificial examples of photomodulation have been demonstrated; however the mechanism of action has just been hypothesised. Figure 1.4 (an adaption of Matt Volgraf's presentation: Switchable Proteins and Channels [2007]) presents a summary of some of the proposed mechanism of action during an artificial action of photomodulation.

Since there is not yet an experimental procedure that confirms the exact *in vitro* configuration of such systems and the mechanism of photomodulation, various postulations have been put forward in various studies. For example, photomodulated enzymes: Concanavalin A and Papain via photochromic dye groups; azobenzenes and spiropyrans have been hypothesised to be via path c) of Figure 1.4 (Willner *et al.*, 1991; 1992). Likewise photomodulation of the enzyme horseradish peroxidase is assumed to take path d) of Figure 1.4 (Weston *et al.*, 1999; Sesay, 2003).

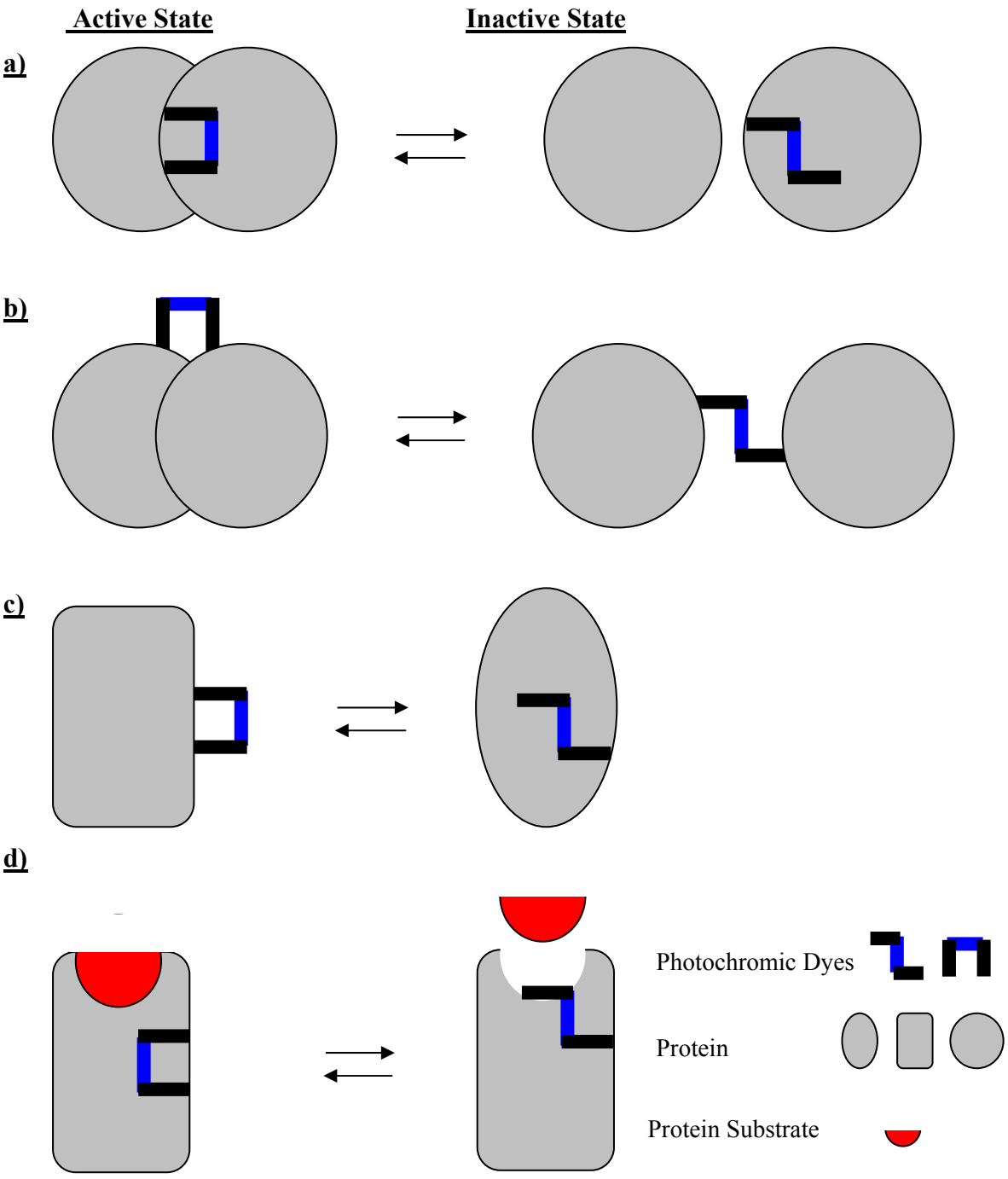


Figure 1.4 : Designing Reversible Protein Photoswitches.

Adapted from Matt Volgraf's presentation: Switchable Proteins and Channels (2007). These are representations of possible modes of molecular photo switch configurations and possible mechanism of modulation.

1.2.4 Examples of Photomodulation of Biological Systems via Photochromic Dyes

1.2.4.1 Introduction

This section describes a number of artificial examples of biological systems that can be photomodulated via photochromic dyes. Photoregulation and photomodulation are two terms used interchangeably by researchers to describe the photo-control of biological activity. A number of research groups have utilised photochromic molecules to modulate a variety of biological systems. As a result, in recent years, research in the area of photochromism has become increasingly important in connection with phenomena other than mere colour change. The occurrences of two different structures which can be reversibly interconverted by means of an external light stimulus are exploited as molecular switches triggered by light aimed to affect an action. Presented are some of the limited examples of photomodulation of biological systems via photochromic dyes, however an extended spiropyran related examples of photomodulation is presented with the spiropyran dye section (Section 1.5.2),

1.2.4.2 Photomodulation of Enzyme Activity

Willner *et al.* conducted a number of enzyme-activity modulation by light. Their work in the 1990s involved the chemical modification of enzymes with photochromic dye groups which were able to modulate the activity of the enzyme by light. Their findings concluded that when the dye-protein complexes are exposed to appropriate wavelength illumination, they inhibit or promote the enzyme activity. For example, the enzyme Concanavalin A was chemically attached to thiophenefulgide (a photochromic dye) (Wilner *et al.,* 1991). The authors were able to alter the enzymes association with 4-nitrophenyl α-D-mannopyranoside, which resulted in ~ 2 fold change in the binding constant upon appropriate illumination. The same enzyme was also studied with tethered spiropyrans and similar results were attained. Papain, a cysteine protease that cleaves the Fc (constant fragment) from the Fab (variable fragment) in antibodies, was covalently attached to azobenzene moieties via the antibody's lysine residues. A change in illumination altered the catalytic activity of

the enzyme. Other successful similar modulation of enzyme activity was possible with horseradish peroxidase, BamHI, and RNase S (Nakayama *et al.*, 2005; James *et al.*, 2001; Weston *et al.*, 1999; Hamachi *et al.*, 1998), whereby the photoswitching capabilities of the attached photochromic dyes resulted in a reversible change in the enzyme activity. In summary a limited variety of enzyme activities have been reversibly photomodulated thus far *i.e.* via chemical modification of the enzyme with photochromic dyes. In other words there has been a successful demonstration of the direct modulation of enzyme activity by previous researchers.

1.2.4.3 Photomodulation of other Biological Systems

Other biological systems have been photomodulated via photochromic dyes. This section presents examples of photomodulation of biological system other than enzyme activity modulation. Sisido *et al.,* (1998) demonstrated a photomodulated antibody – hapten binding reaction. This involved a photochromic hapten, azobenzene, and an anti–trans azobenzene antibody. Upon appropriate illumination, the conversation between the cis and trans azobenzene isomers modulated the antibody – hapten complex formation. This study observed photomodulation of the antigenic activity using photochromic antigens (photochromic dyes that were also antigens in the complex formation).

Photomodulation has also been observed in a modified of *E. coli* by Bose *et al.*, (2006). Azobenzene was attached to an amino acid to form a complex (AzoPhe). AzoPhe was then introduced into the E. coli catabolite activator protein (CAP). CAP regulates a number of catabolite sensitive operons in *E. coli* Binding of cathelicidin antimicrobial peptide to CAP resulted in conformational changes in the protein that increased its binding affinity to its promoter; it resulted in enhanced transcription from CAP-dependent promoters. The binding affinity of CAP was reduced in the presence of AzoPhe upon conversion from trans- to cis- isomers of the dye. It was also suggested that the genetic incorporation of AzoPhe into proteins could be useful to temporarily regulate a variety of biological processes, both *in vitro* and *in vivo*.

The influence of spiropyrans on the transportation of amino acids across cell membranes was studied by Sunamoto *et al.*, (1982). Their findings demonstrated the first example of the photo-controlled transportation of an amino acid across lipid membranes, using a spiropyran embedded in liposomal bilayers of egg phosphatidylcholine. In the presence of light there was a rapid rate of transfer of amino acid across the lipid bilayer. Upon UV irradiation the rate of movement was slowed (reverted back normal rate of transfer).

Muramatsu *et al.*, (2006) demonstrated that genetically and chemically engineered chaperonin azo-GroEL, bearing photoresponsive mechanical gates at the entrance parts of its cylindrical cavity, serves as a semi biological molecular machine with an implemented "AND" logic gate capable of controlling the folding process of proteins in response to ATP and light as input stimuli. This engineered chaperonin trapped denatured green fluorescent protein (GFPdenat) and prohibited its refolding. However hosting azo-GroEL simultaneously detects ATP and UV light, and hence quickly releases GFPdenat to allow its refolding. A 1 min exposure of visible light induced the cis- to trans- isomerisation of the azobenzene groups and a notable retardation of the release of GFPdenat was observed. Muramatsu *et al.*, (2006) believe that such logic gate conceptions based on nanobiotechnologies are important not only for the advancement of molecular machinery but also for integration into the design of secured drug release systems.

Jurt *et al.*, (2006) also demonstrated that a peptide capable of adopting different tertiary structures can be constructed by inserting an azobenzene linker into the polypeptide backbone; a simple transformation (irradiation or heating) can then be used to switch from one form to the other. Their approach complements related efforts to control helix conformation through azobenzene-based photo-crosslinkers between appropriately spaced cysteine side chains.

From the point of view of molecular structure, polypeptides are quite special polymers as they can exist as both disordered and as regularly folded structures typical of those existing in proteins, such as the α-helix and β-sheet. When photochromic molecules

such as spiropyran units are attached to the macromolecular chains, polypeptides appear to respond to light giving large photo induced structural changes (Shimoboji *et al.*, 2002).

1.2.5 Examples of Photomodulation of Non-Biological Systems via Photochromic Dyes

1.2.5.1 Introduction

Photochromic behaviour as previously stated has also attracted a great deal of interest with regards to possible applications in non-biological systems. Like biological systems, synthetic polymers, copolymers and other non biological containing photochromic moieties undergo reversible variations in their structure and conformation upon exposure to different illumination conditions which may also lead to modulation (Pieroni *et al.*, 2001). Presented are some of the limited examples of photomodulation of non-biological systems via photochromic dyes, however an extended spiropyran related examples of photomodulation is presented with the spiropyran dye section (Section 1.5.2),

1.2.5.2 Solubility regulation via Photochromic Dyes

Solubility regulations by photochromic dyes have been more than once been demonstrated to be feasible. Ito *et al.*, (1999) demonstrated that photomodulation of subtilisin solubility can be regulated by a photo-responsive copolymer, a copolymer that carries spiropyran groups on its side chains. Spiropyran-carrying methacrylate covalently attached to subtilisin (= hybrid subtilisin) was found to be completely soluble in toluene and it efficiently catalyzed transesterification. After UV irradiation, the hybrid subtilisin formed a precipitate. The hybrid enzyme was easily and quantitatively recovered by centrifugation. Light irradiation caused a reversal of this effect. Hybrid subtilisin can be repeatedly and reproducibly cycled through solubilisation and precipitation steps without a loss of activity. This study was essentially based on the recovery of the enzyme in a solution rather than its activity modulation.

A similar observation of photoregulation of solubility of polymers containing photochromic moieties was also studied by Arai *et al.* 1996. They linked photochromic spiropyran dye to methyl cellulose to form a complex. Their findings concluded that solubility of the complex in benzene and the contact angle of the complex film with water were reversibly regulated by irradiation with UV light and subsequently with visible light.

Ciardelli *et al.*, (2001) demonstrated that photochromic vinyl polymers, such as polyacrylates containing spiropyran moieties, can undergo photo-induced variations in their viscosity influencing its solubility. They stated that since the viscosity of a polymer system is partly a reflection of polymer conformation, the "photo viscosity effects" were generically attributed to photo-induced conformational changes of the macromolecules.

1.2.5.3 Other Non-Biological Systems Examples

There has been some interest in the use of photochromic dyes as chemical separation agents. Metal ions can be extracted from aqueous solution using organic liquid membranes into which spiropyran dye are dissolved. The merocyanine form of the spiropyran dye is charged and consequently has the ability to attract certain metal ions. This method of chelation extractions is photo reversible (Garcia *et al.*, 2000).

The photo-reversibility of the photochromic dye spiropyran charge has also been applied in metal ion sensors. The development of an optical method to detect and monitor aluminum ions, both clinically and industrially, has been successfully demonstrated by Ren *et al.*, (2007).

Maurer *et al.*, (2005) illustrated another example of photomodulation applied in the concept of a memory device. They developed a photo-chemically controlled photonic-crystal material by covalently attaching a spiropyran derivative to polymerised crystalline colloidal arrays (PCCAs). These PCCAs consisted of colloidal particles that self-assembled into crystalline colloidal arrays (CCAs) when embedded in cross

linked hydrogels. These materials diffract light in UV, visible, or near-IR spectral regions. Excitation of the spiropyran using UV light caused a red-shift diffraction of the PCCAs. Conversely irradiation with visible light caused a blue-shifted diffraction. Thus, this material acts as a memory storage material where information is recorded by illuminating the PCCA and information is recalled and read out by measuring the photonic-crystal diffraction wavelength. The diffraction shifts are resultant from changes in the energy from mixing the PCCA system, as the spiropyran is photo-excited to its different stable forms.

Rosario *et al.*, (2002) explored the design of a photo sensitive surface whose wetting characteristics could be changed by the use of light. A coating of photo-responsive spiropyran molecules covalently bound to a glass surface, along with a mixture of silanes, exhibited reversible changes in wettability when irradiated with UV and visible light. The magnitude of the changes achieved in the contact angle using light was found to be between 11 ° and 14 °. This photo-sensitive coating, applied to the inside walls of capillaries, allowed the flow of water to be induced by light of specific wavelengths i.e. manipulating a microchannel or capillary surface properties using a readily controlled external stimulus.

1.3 Spiropyran Dyes

1.3.1 Overview

An ideal photochromic dye for modulating high-affinity biomolecules function would have the following desirable general characteristics (Hug *et al.*, 1991; Bouas-Laurent *et al.*, 2001):

a) Its photoswitching capability must occur on a relatively faster time scale compared to the observed or monitored reactive nature of the biomolecule.
b) The photochromic dye must have a high quantum yield *i.e.* the measure of the efficiency with which absorbed light produces some effect.

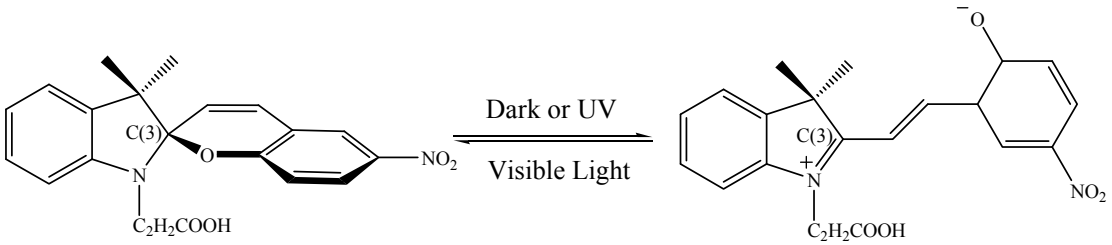
c) The isomers of the photochromic dye must have contrasting physico-chemical properties to aid more definitive switching *i.e.* the chemical / physical properties of the isomers should have distinctively varying effects on the biomolecule and not just a mere colour change.
d) The dye, biomolecule and dye-biomolecular complex should be thermodynamically stable during irradiation with desired wavelength of illumination; they should also be stable at room and at the bimolecular storage temperatures.
e) The dye and the complex formed should have zero or minimal chemical degradation (fatigue) after use; they should also not be fatigued during photochromic-biomolecule complex formation.

As evident in Section 1.2.4, the incorporation of azobenzenes in the photomodulation of biological system has been significantly exploited, largely because of their change in length (Δ length ~ 7Å); geometrical structure upon trans- to cis-photoisomerisation; their simplicity of structure; and to a lesser extent due to their change in dipole moment (Δμ ~3 D) (Banghart *et al.*, 2006). However, spiropyran dyes also have very attractive properties, opening a wealth of exploitable opportunities, and have also been well studied (Banghart *et al.*, 2006). These advantageous properties include:

- The chemical structure of the two isomers of spiropyran dyes are also very distinct, almost appearing as two different molecules. Photochromism of azobenzenes show less differential cis- and trans- properties compared to opened and closed spiropyran dyes.
- Spiropyrans also display the same photoswitching properties as seen in azobenzenes but also have an increase in polarity (dipole moment) upon photo-induction opening of the electrocyclic ring to create the merocyanine form (Δμ ~15 D); This is anticipated to significantly contribute to the modulation phenomena by amplifying the photomodulation effect.

1.3.2 Photochemistry of Spiropyran Dyes

Among many types of organic photochromic compounds, the chemistry of spiro-photochromic compounds has been extensively investigated with special regards to their physicochemical properties. Particular attention has been focused on spiropyrans due to their potential applications in the industrial fields (Li *et al.*, 2004). Spiropyran dyes are unique and have intriguing photochromic proprieties; the isomers undergo colour change, geometrical change, polarity difference and conductivity change (electronic structure) when irradiated with appropriate wavelengths of light (~350mn and > 500nm).



Closed Spiropyran form

Opened Merocyanine form

Figure 1.5 : Schematic of the Photo Isomerisation of the Spiropyran Dye.

The visible light (>500nm) illumination of a carboxylated spiropyran dye (1'-(2-carboxyethy1)-3',3'-trimethyl-6-nitrospiro[2H-1-benzopyran-2,2'-(2H)-indole]) (SP-COOH) in ethanol solution displays a colourless spiropyran closed form. Upon UV illumination (~350nm) or in the dark, the merocyanine opened form is experienced which is highly coloured red. The isomers can be switched back and forth with the appropriate illumination. It should be noted that the dark reaction is a slow conversion rate.

Spiropyran molecules are bicyclical compounds with one atom that is common to both rings. They consist of an extended pyran moiety; and a second moiety which is held in an orthogonal orientation by a common spiro carbon atom (C (3)). The π-electron systems of both constituent halves do not interact because of their orthogonal orientation; as such the absorption spectrum of the compound is essentially the superimposition of the two constituent chromophores (Ernsting *et al.*, 1990).

The spiropyran form is a resonance hybrid of a zwitterionic structure and a neutral structure. When illuminated with UV radiation of about 350nm bond cleavage takes place; the molecule then reconfigures itself into a near planar merocyanine structure. Irradiation of the spiropyran form with UV light causes the formation of an extended π-conjugation merocyanine form by heterolytic cleavage of the spiro C-O bond at the oxygen molecule adjacent to spiro carbon (Shao *et al.*, 2005; Lin, 2003). When this happens the stereogenic spiro centre is lost. This merocyanine form of the dye absorbs light in the visible region of the spectrum due to their extensively conjugated π-electron cloud (Hobley *et al.*, 1999). The zwitterionic structural component is a major contributor to the overall structure of the merocyanine form, as seen in the neutral structure; also the aromaticity of the oxygen-bearing ring is lost. Thus, the molecules may be switched from closed to open forms with UV light, or thermally when in polar solvents; They can conversely be switched from open to closed forms of the dye with visible light, or thermally in non polar solvents, and the stereogenic centre at C (3) is formed again (Eggers *et al.*, 1999).

Spiropyrans have non-polar groups but when converted to merocyanine isomer of the dye they become polar (Ito *et al.*, 1999). The dipole moment of the most stable conformer of the open photo-isomer is known to be about twice the size of the closed form (Cottone *et al.*, 2000). Some spiropyrans can withstand repeated light induced cycling, up to 30,000 cycles in certain cases. They also show good quantum yields for isomerisation (between 10% and 50%), resulting in efficient energy utilization and fewer side reactions. They do not generate free radicals, unlike some other photochemically active molecules (Rosario *et al.*, 2002). Spiropyrans, in their closed form, are soluble in a wide range of organic solvents and generally have relatively lower water solubilities (Garcia *et al.*, 2000).

In non-polar solvents spiropyran molecules generally exhibit normal photochromism and are colourless to pale yellow solutions becoming highly coloured when irradiated with UV radiation; they then revert back to the colourless/pale yellow spiropyran form upon irradiation with visible light. The spiropyran form is a lightly coloured neutral hydrophobic compound, whereas the opened merocyanine compound is

deeply coloured and is more hydrophilic. The position of the established complex equilibrium also depends on many factors; solvent polarity, the nature of the substituent on the molecule, and the concentration of the solution. Although spiropyrans generally show positive photochromism as described, a few spiropyrans, especially those with free hydroxyl, carboxyl or amine groups, exhibit negative photochromism (Zhou *et al.*, 1995).

Since the discovery of photochromic reactions of spiropyrans in 1952 by Fisher and Hirshberg, researchers have investigated spiropyrans for various possible applications a few examples of which were mentioned in section 1.2.4 and in Table 1.1. The transition from spiropyrans to merocyanines has been studied by various groups and has been determined to occur within a time frame of pico- to nano-seconds; thus it is fairly fast compared to the reaction rate of antibodies and enzymes. The mechanism of conversion between isomers has been extensively looked into over the past 50 years and there has been a large volume of research into the conversion of the spiropyran molecule into the merocyanine form. In principle, eight merocyanine conformations are possible, which correspond to different values of the three dihedral angles α, β, and γ describing the rotations around the C8–C10 (N-C=C–C), C10–C11 (C=C–C=C), and C11–C12 (C–C=C–CO) bonds respectively. However, only conformations with a central *trans*- segment (*i.e.* β = 180°) correspond to local energy minima, hence there are only four potentially stable conformations (see Figure 1.6) The equilibrium composition of these four conformations is dictated by the thermodynamics of the solvent system being used (Cottone *et al.*, 2000; Hobley *et al.*, 1999).

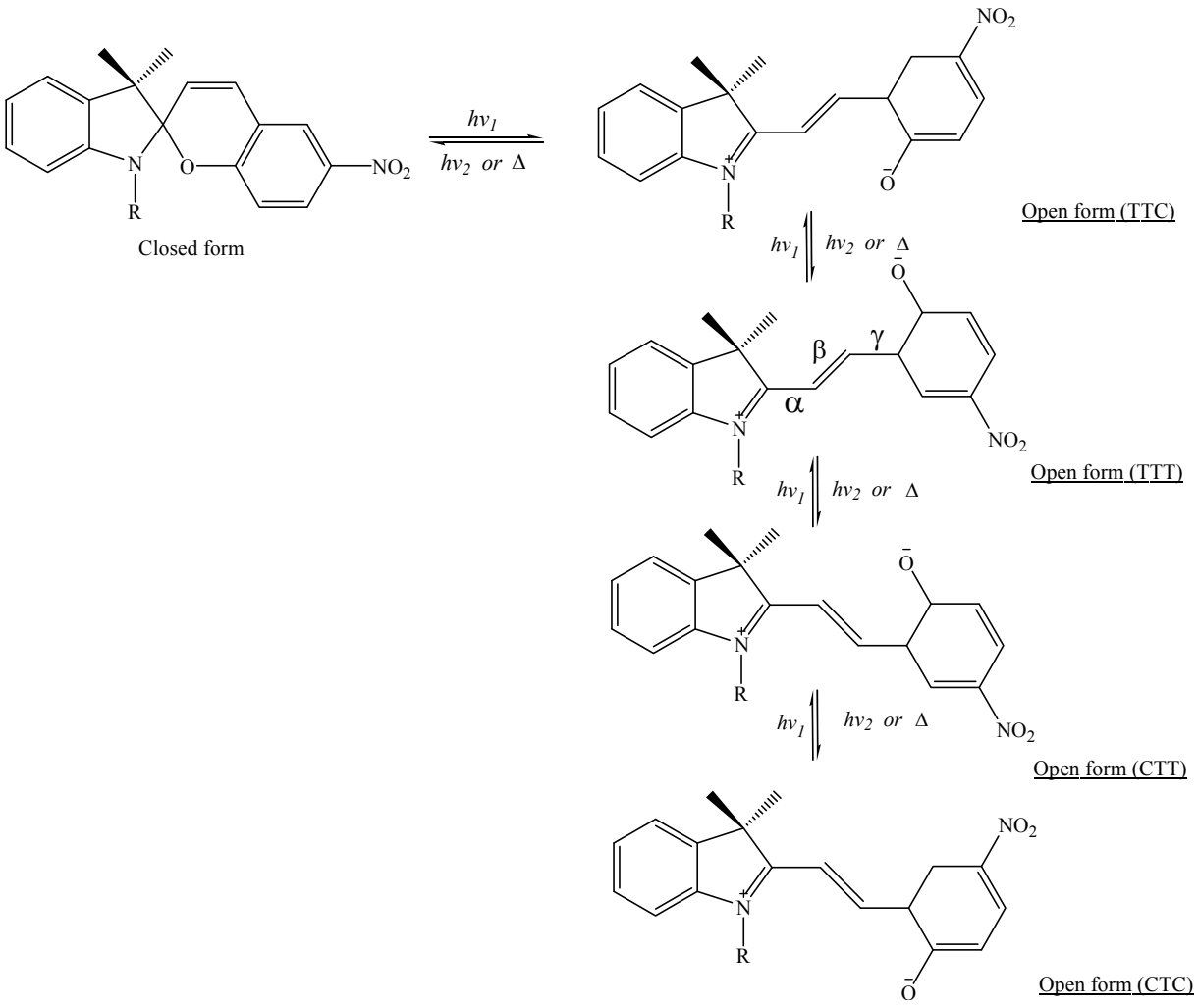


Figure 1.6 : Schematic of the Merocyanine Isomers.

Adapted from Hobley *et al.*, (1999). Formation of the proposed four isomers of the merocyanine forms. T and C denote *trans* and *cis* isomerism.

Only conformations with a central *trans*- segment (*i.e.* β = 180°) correspond to local energy minima (Cottone *et al.*, 2000). It has been suggested that merocyanine form of the spiropyran dye, can potentially exist as any one of the four isomers in Figure1.6 which are all trans- about the central β-bond on the methane bridge, having an equilibrium composition dictated by the thermodynamics of the solvent system being used (Hobley *et al.*, 1999). Merocyanine isomers which are cis- about the central β-bond are high energy isomers and do not significantly contribute to the equilibrium isomeric mixture.

Therefore in theory (as a result of molecular modelling) the most stable merocyanine conformation based on thermodynamics in polar solvents is *trans–trans–cis* or TTC, characterised by $\alpha = 180°$, $\beta \approx 180°$, and $\gamma = 0°$ with CTT, characterised by $\alpha = 0°$, $\beta \approx 180°$, and $\gamma = 180°$. However devising another theoretical modelling using a semi-empirical method (Cottone *et al.*, 2000), it was found out that CTT was the most stable with TTC being the least. The cis-merocyanine and trans-merocyanine form are both considered to be stable, but the conformer distribution may depend significantly on the solvent and substituent attached; as such, there is no general agreement on which structure is the most stable form (Gorner *et al.*, 1998).

1.4 Immunoaffinity Biosensors

> "Biosensors are analytical devices incorporating a biological material (*e.g.* tissue, microorganisms, organelles, cell receptors, enzymes, antibodies, nucleic acids, natural products, etc.), a biologically derived material e.g. recombinant antibodies, engineered proteins, aptamers, etc) or a biomimic (*e.g.* synthetic catalysts, combinatorial ligands, imprinted polymers) intimately associated with or integrated within a physicochemical transducer or transducing microsystem, which may be optical, electrochemical, thermometric, piezoelectric, magnetic or micromechanical" (*Biosensors & Bioelectronics, 2005*).

Biosensors are available in two main formats; biocatalytic and bioaffinity which involve enzyme catalytic technology and immunoaffinity technology respectively. Affinity-based biosensor technology, with its high sensitivity, wide versatility and high throughput, plays a significant role in basic research, pharmaceutical development, and in food and environmental sciences. Likewise, the increasing popularity of biosensors has prompted manufacturers to develop novel instrumentation for recently developed dedicated applications (Baird *et al., 2001*). The biosensors that have been earmarked for large scale production have generally utilised cheap disposable sensors, which are external to the electronics needed to process the signal (Cass *et al*., 1984). However, developing a wide range of disposable sensors has its limitations; not all affinity based sensors can be made available cheaply.

Commercially developed immunosensors, specifically detect analyte in a sample by using an immunoaffinity layer, which is usually the removable component of the device, and are sometimes referred to as sensor chips. Immunoaffinity layers are made by immobilising antibodies or antigens, against a target analyte, on a transducer (a device that converts one type of energy or physical attribute to another for various purposes including measurement or information transfer; which may be electrochemical, optical, magnetic, etc) that produces a detection signal.

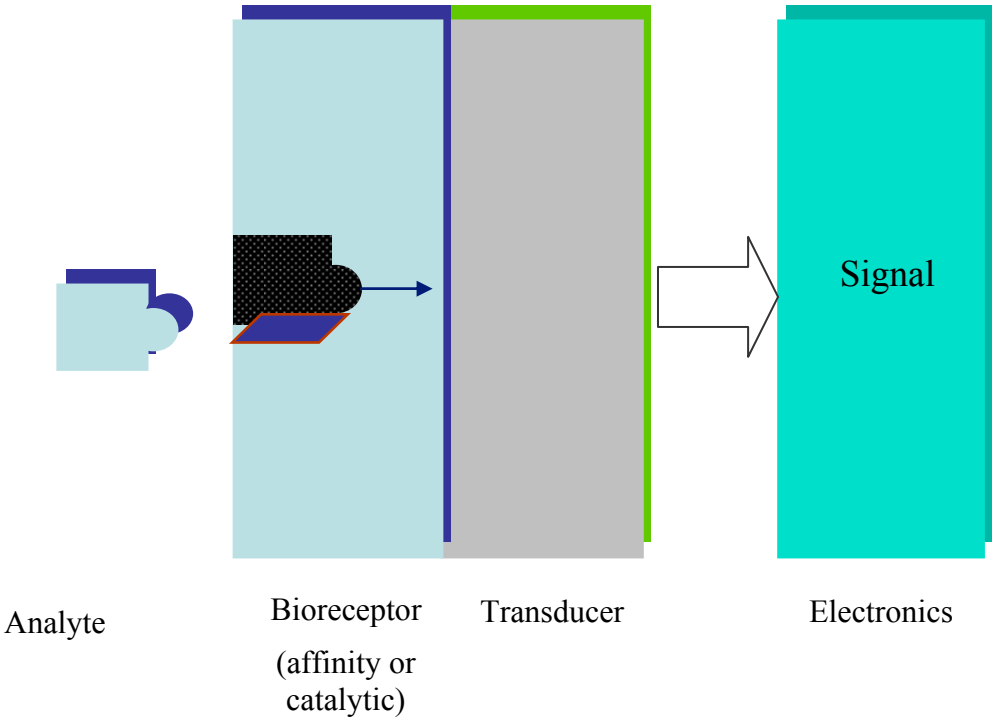


Figure 1.7 : Schematic of Biosensor Technology.

A simplified version of how biosensor technology is generally achieved. Analytes are detected by a specific bioreceptor which is liked unto a transducer. A response or signal is produced which is usually detected electronically.

Affinity-based biosensors have sparked a revolution in the science of biomolecular interaction analysis. The inherent recognition properties of these biomolecules convey a high sensitivity and selectivity to the biosensors. A key advantage of biosensors is that data may be collected in real time, providing detailed information about a binding reaction (Baird *et al., 2001*). This technology forms the heart of an immunosensor device; in order to use immunoaffinity biosensors for successive measurements, the surface has to be free of bound substances.

1.4.1 Immunoaffinity Biosensors Regeneration

For a sensor to be used continuously *in situ* or near point source, the issue of surface regeneration is an important one which must be addressed. Antigen-antibody complexes are highly stable and often require stringent buffer conditions to disrupt binding.

Various regeneration methods to remove bound analyte have been reported. Hitherto different regeneration strategies have been used; the most commonly used methods involve extreme pH buffers, high concentration of surfactants, saturated salt solutions, urea, strong solvents such as 85% ethanol in water and chelating agents Andersson *et al.*, (1999). The use of these "wash" buffers is often unpleasant and aggressive by nature as they are required to disrupt naturally occurring binding events. The regeneration process of the immunoaffinity layer generally results in loss of activity and sensitivity of the layer. This is due to the damage caused by the regeneration buffer used to decouple the affinity complex and / or also as a result of incomplete dissociation of the binding of the detection molecule to its analyte. The use of proteases for regeneration have been reportedly used to regenerate surfaces effectively over 600 times without loss of activity; however other systems have reported as much as 10% decrease in sensitivity with every regeneration of the sensor surface (Wijesuriya *et al.*, 1994). Generally the methods used for renewal of recognition molecules have been both imprecise and overly aggressive towards sensors as well as the analyte. Andersson *et al.*, (1999) suggested a possible solution to the problems faced by the sensing surface during regeneration, which involved tackling the surface binding level rather than the type of reagent used; *i.e.* development of other means of controlling binding affinity. Therefore developing a technology that has the ability to reversibly bind to its affinity partner, via a non-invasive manner, would be highly welcomed.

Light is a naturally occurring stimulus that many biological functions can respond to, depend on and are not necessarily damaged by, at reasonable intensities. Light can offer many advantages, as a means of manipulating systems of either microscopic or macroscopic size, primarily because of the properties of photons. Photons can be applied using extreme spatial and temporal resolutions employing modern laser and light emitting diodes (LEDs) techniques. They are perfectly clean as photons do not leave a residual contamination and, in direct contrast to matter, they do not interact with each other at moderate intensities thus allowing for multiplexing. For these reasons use of molecular assemblies with optical triggers would be an ideal system for the study of molecular processes, as well as the construction of photo-molecular

devices (Renner *et al*., 2005). Control by light can be manipulated very precisely. For example, with the use of lasers and microscopes, rapid jumps in the concentration of the active forms of molecule are possible, while maintaining exact control of the area, time, and dosage.

In 1994 Hohsaka *et al*. were able to demonstrate a reversible association between azobenzene photochromic dye group with monoclonal antibody and an unnatural amino acid. Their findings concluded that the azobenzene group, when in the trans- form, binds to the antibody. When the dye is photo-isomerised to the cis- form the binding ceases and the antibody is released. Kagner *et al*., (1999) demonstrated the feasibility of regenerating a surface plasmon resonance (SPR) biosensor involving antibody – hapten interactions by immobilising a photochromic hapten, dinitospiropyran (DNP) dye, on an SPR surface. The binding of a soluble anti-DNP antibody was modulated by changing the wavelength of surface illumination, which induced conformational changes in the immobilised DNP hapten. This is a simplistic demonstration of a true non-invasive regeneration of bio-sensing system. Both of these findings, although ground breaking with respect to the concept of this study, do not demonstrate a typical photomodulation of an antibody in an active biological system. This is because the photochromic dye represents the antigen in the antigen-antibody complex formation modulation hence its use is limited.

The field of commercial optical affinity biosensors is rapidly evolving, with new systems and detection methods being developed frequently. The possible incorporation of photons, as a driving force, and of photochromic dyes, as molecular switch components, with regards to regeneration in immunoaffinity biosensors elucidates the practicality of nil or minimal side effects of this proposed method.

1.5 Biological Systems to be Employed in Current Study

1.5.1 Introduction

To date the technology of photo-control of antibody affinity via photochromic dyes (spiropyrans) is yet to be fully exhausted. Although there have been various approaches to photomodulate other biological functions as mentioned in section 1.2.4, none of these attempted to apply the phenomenon to antibodies. One possible reason could be because typical antibodies have a molecular weight of approximately 150 kDa; hence they may seem relatively large to photomodulate, especially considering the size of a spiropyran dye (~ 380 mwt) and the number of lysine residues (~ 90: according to entry in structure 1hzh in the RCSB Protein Database) available for coupling on the antibody. Nonetheless, a review of spiropyran related photomodulation may give some insight on the feasibility of control of protein activity.

1.5.2 Published Results of Photomodulation of Some Protein Systems via Spiropyran Dye Derivatives

There has been a limited number of applications of spiropyran dyes to photomodulate the activity of proteins via chemical modification of the protein with the dye. A history of successful modulation of protein function by spiropyran dyes was looked into, in an attempt to establish trends in biological systems modulation by the spiropyrans. In addition to the generic examples of photomodulation described in section 1.2.4, photomodulation of proteins via spiropyran dyes is illustrated in Table 1.1. It is important to note that the illustration in Table 1.1 shows that photomodulation of protein activity, aided by spiropyrans, does not necessarily follow a general trend; as in which dye isomer promotes modulation; however details of some experimental conditions were unknown and this may have a significant effect on the photoswitching mechanism of the attached dye.

Table 1.1 : Published Results of Photomodulation of Some Protein Systems.

These are examples of photomodulation of proteins via spiropyran dye derivatives. The protein and it molecular weight is shown for comparison, also the coupling chemistry and how irradiation affects modulation.

Coupling Chemistry	Protein / Molecular Weight (kDa)	Solvent	Irradiation Condition	Modulation Details	Reference
EDC/NHS	Subtilisin / ~ 27	During coupling, Dye is in Toluene and Protein is in water. Activity of modified enzyme reaction in an unspecified organic solvent	UV & Visible Light	UV (dye: merocyanine form) = modified protein becomes insoluble Visible Light (dye: spiropyran form) = modified protein becomes soluble	Ito (1999)
EDC/NHS	Glucose Oxidase / ~ 150	Coupling in Hepes Buffer (pH 6.5) Experiment in Water/Ethanol (1:10)	UV & Visible Light	UV (dye: merocyanine form) = modified protein is restored near native activity Visible Light (dye: spiropyran form) = modified protein process lower enzyme activity	Willner (1997)
EDC/NHS	HRP / ~ 44	MES (pH 6.0) / Ethanol (5:1)	UV & Visible Light	UV (dye: merocyanine form) = modified enzyme activity is restored near native activity Visible Light (dye: spiropyran form) = decrease in modified enzyme activity	Weston (1999) Sesey (2003)

Coupling Chemistry	Protein / Molecular Weight (kDa)	Solvent	Irradiation Condition	Modulation Details	Reference
	Poly (L-Glutamic Acid) / ~ 40	Hexafluoro-2-propanol (HFP)	Dark & Visible Light	Dark (dye: merocyanine form) = decrease of the helix content and recovery of the original disordered conformation Visible Light (dye: spiropyran form) = Resulted in a α-helix ordered structure of polypeptide *modified protein exhibit negative photochromism*	Ciardelli (1989) Pieroni (2002)
DCC	Amylase / ~ 96	Unspecified Organic Solvent	Dark & Visible Light	Dark (dye: merocyanine form) = decrease in modified enzyme activity Visible Light (dye: spiropyran form) = modified enzyme activity is restored near native activity	Aizawa (1977) Namaba (1975)
PPDs/ DMAPN	Chymotrypsin / ~ 25		UV & Visible Light	UV (dye: merocyanine form) = decrease in modified enzyme activity Visible Light (dye: spiropyran form) = modified enzyme activity is restored near native activity	Willner (1993)
EDC	Concanvalin A / ~ 37	Phosphate buffer, 0.1 M (pH= 8)	UV & Visible Light	UV (dye: merocyanine form) = decrease in modified enzyme activity Visible Light (dye: spiropyran form) = modified enzyme activity is restored near native activity	Zahavy (1994) Willner (1993)

Coupling Chemistry	Protein / Molecular Weight (kDa)	Solvent	Irradiation Condition	Modulation Details	Reference
Direct (Dye embedded in bilayer)	Liposomal bilayers of egg phosphatidylcholine / n/a	Apolar solvents used (hexane and octanol buffered at pH 6.0)	Dark & Visible Light	Dark (dye: merocyanine form) = Protein was not permeable in modified bilayer Visible Light (dye: spiropyran form) = Protein permeable in modified bilayer	Sunamoto (1982)
Thiol coupling	*Polymer-Protein* *Protein:* Streptavidin / ~ 53 *Polymer: N,N*-dimethylacrylamide (DMA)-*co*-4-phenylazophenyl acrylate (AZAA)	Ethanol / Dimethylformamide (DMF)	UV & Visible Light	UV (dye: merocyanine form) = modified polymer - protein is soluble Visible Light (dye: spiropyran form) = modified polymer - protein is insoluble	Shimoboji (2002)
Thiol coupling	*Polymer-Protein* *Protein:* Streptavidin / ~ 53 *Polymer:* DMA-*co*-*N*-4-phenylazophenyl acrylamide (AZAAm)	Ethanol / Dimethylformamide (DMF)	UV & Visible Light	UV (dye: merocyanine form) = modified polymer - protein is insoluble Visible Light (dye: spiropyran form) = modified polymer - protein is soluble	Shimoboji (2002)

The results of the published work in Table 1.1 demonstrate the effect of photomodulation varies from protein to protein. A typical example, illustrating this variation is the work undertaken by Shimoboji *et al*., (2002); in this a change in incorporated polymer type resulted in an inversion of the photomodulation effects *i.e.* the form of dye responsible for one effect (solubility) in one polymer was responsible for the opposite effect (insolubility) when the polymer associated was slightly

changed. This implies that the dye can react differently to different target molecules, even if they are similar.

Additionally some research groups stated that the modulation can be driven by a dye-protein complex under UV illumination and reversed by visible light irradiation application (or vice versa); other groups failed to mention UV illumination and opted for the dark adaptation / visible light mechanism of photoswitching. Dark adaptation is known to be a slower mechanism of photomodulation; hence it may be considered that some photoswitching mechanisms were only viable via dark adaption and visible light illumination (Sunamoto *et al.*, 1982; Ciardelli *et al.*, 1989).

The medium of reaction also varied with some experiments being conducted in organic medium while others were undertaken in aqueous medium, with further experiments being carried out in a combination of both media at varying ratios. A speculative reason for solvent choice may be that certain solvents favoured photoswitching of attached dye under UV/visible light illumination (despite possible denaturation of the protein).

On the basis of protein molecular size, it can be noted (with the exception of Glucose oxidase modulation by Willner *et al.*, (1997)), that none quite are as large as the average size of an antibody (150 kDa); however the modulation of Glucose oxidase signifies that the size of the protein may not necessarily be as major a factor as previously thought, and possibly the availability of lysine groups for coupling that will aid photomodulation. With regards to coupling chemistry, it is thought that the variation in choice was probably dependant on preference within each group; however most utilised carboxyl-lysine group coupled via carbodiimides.

1.5.3 Reason Behind Choice of Biological Systems to be Studied

This study aims to develop a chemically modified antibody (by spiropyran dyes) whose affinity can be controlled by the irradiation with photons. However, antibodies are not cheap hence it would be ideal to set up experimental procedures with a cheap

protein and apply the finding on the antibodies. HRP was chosen not only for ease of exploitation, *i.e.* to aid the set up of an adaptable experimental protocol for antibody photomodulation since HRP has been previously modulated by others (Weston et al., 1999; Sesay, 2003); HRP was also chosen as it is a readily available enzyme and its activity can be assayed using colourometric reagents readily available. Additionally HRP has a molecular size similar to an antibody fragment (~ 44 kDa). This further exploitation may also give an insight into whether it is the size of protein or number of available lysine groups that has a significant effect on photomodulation.

1.5.4 Horseradish Peroxidase

Horseradish peroxidase (HRP) is isolated from horseradish roots (*Amoracia rusticana*) and belongs to the ferroprotoporphyrin group of peroxidases. Its full nomenclature is: *hydrogen-peroxide oxidoreductase, EC 1.11.1.7*. HRP is a single chain polypeptide containing four disulfide bridges. It is a glycoprotein containing 18% carbohydrate. The carbohydrate composition consists of galactose, arabinose, xylose, fructose, mannose, mannosamine, and galactosamine depending upon the specific isozyme. Its molecular weight is ~ 44 kDa (Zollner, 1993; Shannon *et al*., 1966; Welinder, 1979). It possesses ~ 12 lysine residues, according to the entry in structure 3atj in the RCSB protein database. This is relevant with respect to its application in the study in which lysine residues will be utilised in coupling carboxylated spiropyran dyes to carboxyl groups.

HRP readily combines with hydrogen peroxide (H_2O_2) and the resultant complex [HRP–H_2O_2] can oxidize a wide variety of chromogenic hydrogen donors. HRP produces a distinctive colour when exposed to an appropriate solution. It can also utilize chemi-luminescent substrates such as luminol and isoluminol and fluorogenic substrates such as tyramine, homovanillic acid, and 4–hydroxyphenyl acetic acid (Schomberg *et al.*, 1993). HRP is however inhibited by the following compounds: sodium azide, cyanide, L–cystine, dichromate, ethylenethiourea, hydroxylamine, sulfide, vanadate, p–aminobenzoic acid, Cd^{2+}, Co^{2+}, Cu^{2+}, Fe^{3+}, Mn^{2+}, Ni^{2+}, Pb^{2+} (Schomberg *et al.*, 1993). The optimum pH of HRP activity is in the range of 6.0 to

6.5; activity at pH 7.5 is 84% of the maximum. The enzyme is most stable at pH within the range of 5.0 to 9.0 (Deshpande, 1996). HRP has a vast number of applied uses including being a label for antibody-antigen complexes, a cytochemical marker in immunohistochemical staining and as a tracer to follows the course of individual neurons as it is transported by most neural pathways (Deshpande, 1996).

1.5.5 Antibody

1.5.5.1 Antibody Structure

Antibodies are glycoproteins that are found in blood and tissue fluids, as well as many other secretions. Structurally they are globulins (in the γ-region of protein electrophoresis). An antibody can be monomeric (the basic structure represented below), dimeric, trimeric, tetrameric, pentameric, etc. The monomer is a "Y"-shape molecule (Figure 1.8) that consists of two identical heavy chains and two identical light chains connected by disulfide bonds (Muyldermans, 2001). Antibodies are produced by a kind of white blood cell (B cell). There are several different types of antibody heavy chains and several different kinds of antibodies, which are grouped into different isotypes based on which heavy chain they possess. Five different antibody isotypes are known in mammals which perform different roles and help direct the appropriate immune response for each different type of foreign object they encounter (Market *et al*., 2003). These five isotypes of heavy chain are γ, δ, α, μ and ε. The type of heavy chain present defines the class of antibody; these chains are found in IgA, IgD, IgE, IgG, and IgM antibodies, respectively.

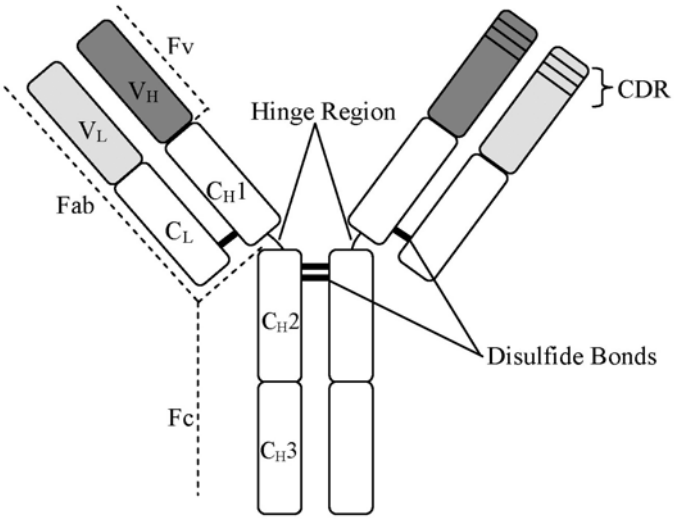


Figure 1.8 : Structure of an IgG Antibody.

Relative positions of the variable domains of the light (V_L) and heavy (V_H) chains which together make an Fv region. Digestion with papain enzyme breaks the IgG molecule in the hinge region before the H-H inter-chain disulfide bond results in the formation of Fab region which has two identical fragments that contain the light chain (V_L and C_L domains) and the heavy chain (V_H and C_H1). The remainder of the two heavy chains each containing a C_H2 and C_H3 domain make up the Fc region. The complementarity determining region (CDR) is located at the tips of the variable domains. Carbohydrates (not shown in this diagram) are attached to the C_H2 domain in most immunoglobulins. However, in some cases carbohydrates may also be attached at other locations.

Heavy chains α and γ have approximately 450 amino acids; while μ and ε have approximately 550 amino acids (Pier *et al*., 2004; Janeway *et al*., 2005). Each heavy chain has a constant region, which is the same by all immunoglobulins of the same class; and a variable region, which differs between immunoglobulins of different B cells, but is the same for all immunoglobulins produced by the same B cell. Heavy chains γ, α and δ have the constant region composed of three domains but have a hinge region; the constant region of heavy chains μ and ε is composed of four domains. The variable domain of any heavy chain is composed of one domain. These domains are about 110 amino acids long. There are also some amino acids between constant domains. There are only two types of light chain: λ (lambda) and κ (kappa). In humans, they are similar, but only one type is present in each antibody. Each light chain has two successive domains: one constant and one variable domain. The approximate length of a light chain is from 211 to 217 amino acids. Additionally the

tip of the variable domain is extremely reactive, allowing millions of antibodies with slightly different structure of the tip to exist. This region is known as the hypervariable region (or complementarity determining region, CDR). Along with class switching, random combinations of gene segments that encode different antigen binding sites allows production of a large number of diverse antibodies.

1.5.5.2 Antibody Function

Antibodies function to eliminate the antigen that elicited their production, usually independently of the particular class of antibody. These functions reflect the antigen binding capacity of the molecule as defined by the variable region and CDR. For example, an antibody might bind to a toxin and prevent that toxin from entering host cells where its biological effects would be activated. Similarly, a different antibody might bind to the surface of a virus and prevent that virus from entering its host cell. In contrast, other antibody functions are dependent upon the immunoglobulin class. These functions are contained within the constant regions of the molecule. For example, only IgG and IgM antibodies have the ability to interact with and initiate the complement cascade. Likewise, only IgG molecules can bind to the surface of macrophages via Fc receptors to promote and enhance phagocytosis (Pier *et al*., 2004).

1.5.5.3 Monoclonal Antibodies

Monoclonal antibodies are identical antibodies produced by the same type of immune cell, derived from clones of a single parent cell. It is, theoretically, possible to create monoclonal antibodies, to almost any substance, that specifically bind to that substance; they can as such be used to detect or purify that substance. Monoclonal antibodies enable perpetual production of antibodies which react with a single specific epitope. They have become an important tool in biochemistry, molecular biology and medicine since they became widely available in the 1980's (Pier *et al*., 2004).

Monoclonal antibodies are highly specific - due to their nature of originating from one parent clone. Monoclonals recognise only one epitope of the antigen, and thus will usually give substantially less background. When compared to the heterogeneity of polyclonal antibodies, the homogeneity of monoclonal antibodies is very high. If experimental conditions are kept constant, using monoclonal antibodies will make results consistently reproducible between experimental replicates due to the clonal nature of monoclonal antibodies. Their specificity also makes them extremely efficient for the binding of antigen within a mixture of related molecules, such as in the case of affinity purification.

1.5.5.4 Polyclonal Antibodies

In contrast to monoclonal antibodies, polyclonal antibodies are antibodies that are derived from many different cells or cell lines, similar to the mixture of antibodies found in sera. Polyclonal antibodies are therefore a mixture of different specificities. This is in contrast to monoclonal antibodies which are derived from one clone. Polyclonal antibodies are derived simply from the injection of an antigen into an animal (typically but not exclusively mouse, rabbit, goat or sheep) Janeway *et al*., 2005). The result is a mix of antibodies reacting to, perhaps, several epitopes on the antigen, hence this batch of antibodies may be less specific when compared to monoclonals.

Polyclonals are inexpensive to produce when compared to the cost of monoclonal antibody technology. In addition, large quantities of polyclonal antibodies (~10 mg/ml) can be produced from the serum of an immunised animal. There are also advantages to the use of polyclonal antibodies from a scientific perspective. Because polyclonal antibodies contain the entire antigen-specific antibody population, they offer a statistically relevant glimpse into the overall picture of an immune response (Janeway *et al*., 2005).

1.5.6 Antibody Fragments

Genetic engineering of intact immunoglobulins permits the expression of recombinant antibody fragments, made up of only the variable regions of both the heavy and light chains (Strachan *et al*., 1998). Antibody fragments produced by proteolytic digestion have proven to be very useful in elucidating structure/function relationships in immunoglobulins (Xiang *et al*., 2007). Recombinant antibodies application in biotechnology and medicine are on the increase. This may be because the active molecules can offer significant advantages over whole antibodies such as lower overall COGs (cost of goods); target specificity with greater flexibility; avoid undesirable effects of the Fc region and specific multivalent species can be easily engineered. A variety of antibody formats have been employed, which reflect differences in the production method as well as the intended use (Worm *et al*., 2001).

The Fab fragment of an antibody is a structurally independent unit that contains the antigen-binding site; it is thought that its stability is not influenced by the Fc region, as it is separated by the hinge region (Figure 1.8). In some cases a fragmented antibody can be made that binds to an antigen but does not mediate the effector functions of antibodies. These are $F(ab')_2$. They are divalent and during preparation the Fc region of the molecule is digested into small peptides by pepsin. The four domains of the Fab fragment (V_H, C_H1, V_L, C_L) interact through a large interface between the chains (V_H/V_L and C_H1/C_L) and a small one between the variable and constant domains (V_H/C_H1 and V_L/C_L) of each chain (Worm *et al*., 2001). Hence covalent fusion has been engineered to increase stability.

Due to the much smaller size of the antibody fragment in comparison to the intact antibody, antibody fragments provide a means of more efficient binding and capturing of small molecular weight organic targets. They can be easily made in a suitable microbial host such as *Escherichia coli*, and retain a binding capacity similar to that of the parent antibody molecule. In addition, antibody fragments can also be made and expressed in eukaryotic systems, including transgenic plants (Strachan *et al*., 1998).

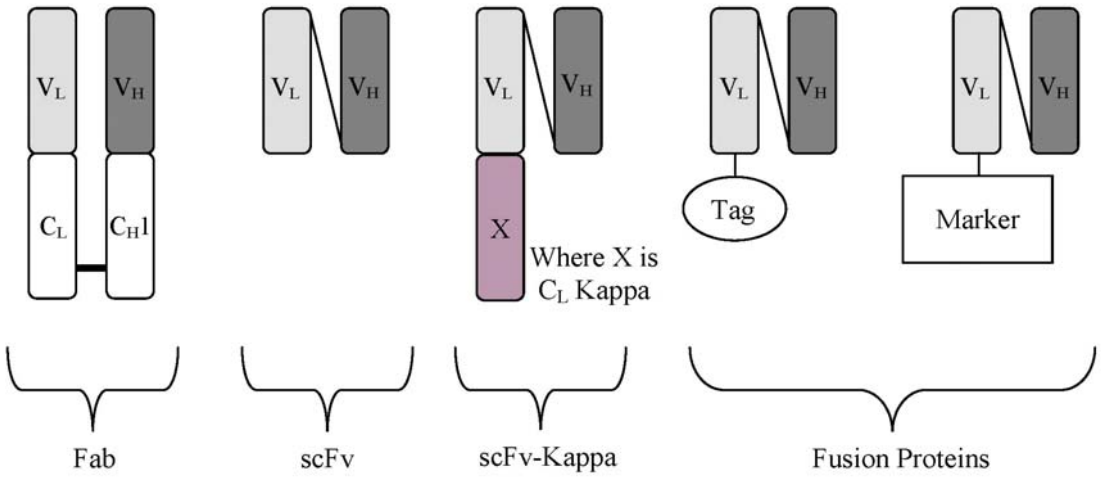


Figure 1.9 : Schematic Representation of Antibody Fragments.

The structural definitions are linked with the general antibody structure in Figure 1.8. Fragments are formed via enzyme cleavage of the variable regions of both the heavy and light chains and in some cases (Fab fragments), part of the constant chains.

The antigen binding ability of an antibody is usually conserved, even when only the V_H and V_L domains are used. Single-chain Fv antibodies (scFv) have been reported to be more stable than Fab antibody fragments when intracellularly expressed in eucaryotic cells. However, when positive Fab clones from a Fab-antibody combinatory library was changed into the scFv format, very low expression levels were experienced (Fredholm *et al*., 1996). One production method by Fredholm *et al*., (1996) and Hayhurst *et al*., (1996) established that when the antibody format for one clone was changed, by the addition of the C kappa domain downstream of the original scFv, a substantial increase in antibody expression levels in the bacterial system was noted. The fragment, scFv-kappa, has an approximate molecular mass of about 44 kDa.

This method of production, second generation, single chain antibody fragment scAbs are sometimes classified as fusion proteins since tags and makers can also be linked with the antibody fragment as desired, illustrated in Figure 1.9. These second generation single chain antibodies can be produced rapidly in *Escherichia coli*,

providing valuable reagents without the need for animal immunisation (Goldman *et al*., 2003).

1.5.6.1 Antigen-Antibody Interactions

An antigen (Ag) may be described as a molecule which elicits a specific immune response when introduced into an animal. More specifically, antigenic (immunogenic) substances are:

- Generally large molecules (>10,000 Daltons in molecular weight),
- Structurally complex (proteins are usually very antigenic),
- Accessible (the immune system must be able to contact the molecule), and
- Foreign (not recognizable as "self").

Antibodies (Ab) are specifically produced in response to an antigen and bind to it via non-covalent interactions. Antigen binding by antibodies is the primary function of antibodies and is their role in the protection of their host organism.
The antigenic determinant/ epitope (a unique part of the antigen recognised by an antibody) allows antibodies to identify and bind only their unique antigen in the midst of other different molecules in a medium. Some antibodies can bind to few closely related antigens which all contain the same epitope e.g. a group of related targets with a common structural feature. The valency of an antibody (the number of antigenic determinants that an individual antibody molecule can bind) is known to be at least two and in some instances more.

Interactions between antigen and antibody involve non-covalent binding of the epitope to the variable region (CDR) of both the heavy and light immunoglobulin chains. These interactions are analogous to those observed in enzyme-substrate interactions and they can be defined similarly. To describe the strength of the antigen-antibody interaction, one can define the affinity constant (K_a) as:

$$\text{Affinity } K_a = \frac{[Ab - Ag]}{[Ab] \times [Ag]} = 10^4 \text{ to } 10^{12} \text{ L/mol}$$

If the interaction between antigen and antibody was completely random, it would be expected the concentrations of free antigen, free antibody and bound Ag-Ab complex to be equivalent. In other words,

$$\text{Affinity } K_a = \frac{1}{1 \times 1} = 10^0 \text{ L/mol}$$

Therefore, the greater the value of K_a, the stronger the affinity between antigen and antibody.

1.6 Ionic Liquids

Ionic liquids are novel materials that have a number of unique but important properties such as high polarity, non-flammability, low melting point (<100 °C), chemical and thermal stability, negligible vapour pressure, consist of loosely co-ordinating bulky ions and remain as liquids within a broad temperature window (liquid window of up to 300 ºC enabling wide kinetic control) (Yang *et al.,* 2005; Abbott *et al*., 2001). Generally they are organic salts, which are liquid at ambient temperature, and solubilise a great number of compounds such as organic solvents (especially when they are polar, e.g. dichloromethane and tetrahydrofuran), inorganic solvents and polymeric compounds (Kaar *et al.,* 2003).

Ionic liquids or sometimes referred to as room temperature molten salts, when immiscible, can form biphasic systems with classical solvents (Mori *et al*., 2005). They can also be used as non-aqueous polar alternatives for phase transfer processes. These ambient temperature ionic liquids have been proposed as possible solvents for many applications including electrodeposition, synthesis and batteries. (Welton, 1999; Wasserscheid *et al.,* 2003).

In contrast to traditional solvents, ionic liquids are composed of ions. They are viscous, denoted by the strength of the Van der Waals interactions, and have the tendency to form hydrogen bonding as a result of dipole-dipole interactions that can exist between its electronegative atoms and nearby hydrogen atom or other cation (Yang *et al.,* 2005). A major advantage of ionic liquids, which is not the main subject matter of this current study, is the ability to fine tune ionic liquids by altering the cation, anion and attached constituents to manipulate the solvent's physical and chemical properties.

Ionic liquids are generally based on quaternary ammonium salts, with the majority of previous work being carried out on imidazolium and pyridinium cations. A range of large anions have been used and these can be classified as either metal containing, e.g. $Al_2Cl_7^-$, or non-metal containing e.g. PF_6^- or $(CF_3SO_2)_2N^-$. The principle behind the

low freezing point of the salts is that the ions are large and non-centrosymmetric and therefore pack less easily into a lattice.

Deep Eutectic Solvent (DES) is a class of ionic liquids which are exploited in this study. DES are ambient temperature ionic liquids formed by a mixture of quaternary ammonium salts with hydrogen donors such as amines and carboxylic acids, that result in low freezing point much lower than either of the individual components, rendering them effectively simple eutectics. The deep eutectic phenomenon was first described by Abbott *et al.*, (2001) which involved 1:2 molar mixture of choline chloride (2-hydroxyethyl-trimethylammonium chloride) and urea. Choline chloride has a melting point of 302 °C and that of urea is 133 °C. The eutectic mixture however melts as low as 12 °C. The physical properties such as viscosity, conductivity, and surface tension of these DES are similar to ambient temperature ionic liquids.

1.6.1 Ionic Liquids and Biological Systems

Over the past decade, ionic liquids have been increasingly employed as substitutes for the traditional organic solvents in chemical reactions. Its zero vapour pressure is a desired property for exploitation in biological systems. The charged components of ionic liquids result in a strong charge interaction that effectively results in a zero vapour pressure, hence ionic liquids do not evaporate and are even stable as liquids in a vacuum. To date, biological applications of ionic liquids have been limited to demonstration of enzyme activity in such solvents. Enzyme reactions studied in ionic liquids and have shown to enhance activity, stability and selectivity for a number enzymes (Yang *et al.*, 2005). Antigen-antibody interactions are yet to be performed in an ionic liquid environment. In a growing number of situations it is desirable for biomolecules to function when associated with surface layers and where the surfaces are not in contact with bulk aqueous media but with gaseous media. The feasibility of biological function such as antigen-antibody interactions will pave the way for new technologies as well as widen the scope of antigen-antibody application.

1.7 Conclusions Drawn from the Literature Review

Affinity-based biosensors face an issue with regeneration of antibodies employed. The current regeneration strategies commonly used, such as extreme pH buffers, high concentration of surfactants, saturated salt solutions, detergents, chelating agents, etc. are unpleasant and aggressive. They can disrupt the binding environment resulting in loss of activity and sensitivity of the immunoaffinity layer. Generally the methods used to date for renewal of recognition molecules have been both imprecise and overly aggressive towards sensors.

Photochromic dyes have been utilised to photomodulate biological systems and non-biological systems for over three decades. This has prompted the study of a number of photochromic dyes. Currently azobenzenes have been exploited with regards to photomodulation of enzymes, however, spiropyran dyes seem to have a number of advantages over azobenzenes, in particular that they can exist in two very distinctive definite ordered structures with varying polarity properties (Ciardelli *et al.*, 2001). These added benefits are believed to have a more substantial effect, with regards to its application in biological system photomodulation.

Artificial photomodulation of antigen-antibody reactions has been attempted by limited number of researchers. Kagner *et al*., (1999) demonstrated how an immobilised photochromic dye (dinitospiropyran), acting as an antigen component to a Surface Plasmon Resonance (SPR) gold surface. Sisido *et al*., (1998) also demonstrated photomodulation of antigen activity in an antigen-antibody coupling reaction. Hohsaka *et al*., (1994) demonstrated the reversible association of azobenzene photochromic dye group with a monoclonal antibody against an unnatural amino acid. However, the modification of an antibody whose affinity could be photomodulated has yet to be established. The development of a photochromic antibody, whose affinity can be controlled via photons, will result in a less aggressive antibody regeneration technique when applied to an affinity based biosensors.

Ionic liquids, as an alternative to traditional solvents, have very interesting properties that gives them some advantages in certain extreme. Currently, enzymatic reactions are the only biological systems known to be feasible in ionic liquids. The incorporation of antigen-antibody reactions into ionic liquids will aim to widen the application of antigen-antibody reactions. This will ultimately be of additional benefit if also applicable to photochromic antibodies.

1.8 Aims and Objectives

The main aim of this project is to establish the feasibility of "artificial antibody affinity control by illumination" (*i.e.* "*on* and *off*" switchable photochromic antibodies) and to attempt to develop an understanding of the mechanism of affinity modulation.

- The primary objective is to synthesize photochromic dyes (spiropyrans) which are capable of being covalently conjugated to proteins, via a carbodiimide mediated reaction. Dye characterisation and its physicochemical properties in various environments are also to be observed.

- The next objective is to utilise the synthesised dye to photomodulate Horseradish peroxidase (HRP). As a model system, the technologies for fabricating an optical molecular switch for the enzyme (HRP) will be used to develop methods.

- The third objective is to develop chemically modified antibodies whose affinity can be influenced *i.e.* controlled by the effect of different wavelengths illumination. This is be achieved by covalently attaching the synthesised spiropyran dyes to the antibodies. The reversible property / structural change experienced by spiropyran dyes upon exposure to different wavelengths illumination is anticipated to reversibly distort and recover the structure of the attached protein and as a result, have an influence on its affinity as a switch. The overall development and evaluation of the application of spiropyran dyes as optical switches for antibodies may contribute towards advancement in biosensor technology.

- The final objective is to ascertain the feasibility of antigen-antibody reactions in ionic liquids and assess the potential in obtaining photochromic antibodies in ionic liquids.

Chapter 2

SYNTHESIS AND CHARACTERISATION OF CARBOXYLATED SPIROPYRAN DYES

2 Synthesis and Characterisation of Carboxylated Spiropyran Dyes

2.1 Introduction

The main objective of the work reported in this chapter is the synthesis and characterisation of spiropyran dyes. Spiropyrans dyes as described in section 1.3.2 are photochromic dyes. The base structure of a spiropyran dye molecule (1',3'-dihydro-1',3',3'-trimethyl-6-nitrospiro[2H-1-benzopyran-2,2'-(2H)-indole]) is illustrated in Table 2.1. This is also the basic form of the dye which can be commercially purchased, hence forth will be referred to as SP-Sigma in this literature.

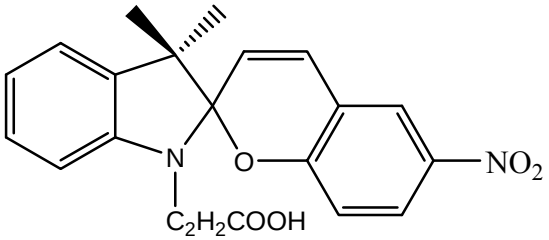
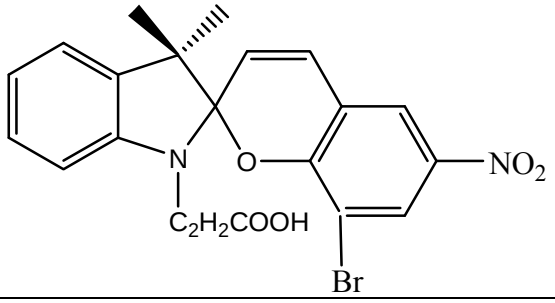
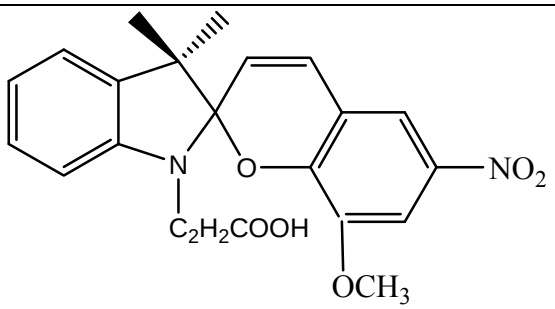
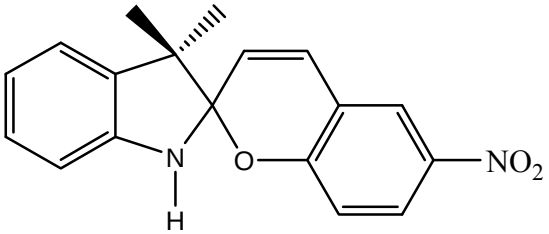
To covalently attach the dye to an amine group of a protein, further modification is required, specifically the synthesis of a carboxylated version of the core spiropyran molecule. Aizawa *et al*., (1977) first established the method of treating 2,3,3-trimethylindolenine with 3-iodopropionic acid to obtain l-(2-carboxyethyl)-2,3,3-trimethylindolenium iodide (CE-TMI-I), and subsequent treatment of the CE-TMI-I with 5-nitrosalicylaldehyde in the presence of piperidine gives 1'-(2-carboxyethy1)-3',3'-trimethyl-6-nitrospiro[2H-1-benzopyran-2,2'-(2H)-indole, the carboxylated spiropyran dye denoted as SP-COOH in this thesis (see Table 2.1) – figure 2.1 shows a schematic representation of the synthesis of the carboxylated spiropyran dye. The carboxylic acid side chain allows the use of water soluble conjugation chemistry such as EDC-NHS to enable straightforward conjugation of proteins with spiropyrans.

Changing the substituents on the chromophore of the spiropyran dye is known to lead to altered wavelength sensitivity and also may affects the thermal stability and extent of photo conversion between isomers. For this reason, a significant challenge remains in designing a photoswitch that performs to its maximum potential (Banghart *et al.*, 2006). As a result a variety carboxylated spiropyran dyes were considered. The inclusion of derivatives of SP-COOH (SP-COOH-Me and SP-COOH-Br) (see Table 2.1) were selected because of readily available chemical intermediates needed for synthesis; likewise the method of synthesis was very similar SP-COOH. Methoxy substituents are known to generally increase aqueous solubility of the compound

attached to it. They are also electron-donating groups and can cause an organic compound to become less acidic.

Table 2.1 : Derivatives of Spiropyran Dyes in Current Study.

Structural representation of the spiropyran dyes synthesised or obtained in this study.

	1'-(2-carboxyethy1)-3',3'-trimethyl-6-nitrospiro[2H-1-benzopyran-2,2'-(2H)-indole **SP-COOH** (Synthesised for Study)
	1'-(2-carboxyethy1) -8-bromo-3',3'-trimethyl-6-nitrospiro[2H-1-benzopyran-2,2'-(2H)-indole **SP-COOH-Br** (Synthesised for Study)
	1'-(2-carboxyethy1) -8-methoxy-3',3'-trimethyl-6-nitrospiro[2H-1-benzopyran-2,2'-(2H)-indole **SP-COOH-Me** (Synthesised for Study)
	1',3'-Dihydro-1',3',3'-trimethyl-6-nitrospiro[2H-1-benzopyran-2,2'-(2H)-indole] **SP-Sigma** (Purchased for Study)

2.2 Chemicals, Materials and Equipment

The following chemical consumables were purchased from Sigma Aldrich, UK: 3-Iodopropionic Acid (Sigma Code: I10457), 2,3,3-Trimethylindolenine (Sigma Code: T76805), 5-Nitrosalicylaldehyde (Sigma Code: 55967), 3-Methoxy-5-Nitrosalicylaldehyde (Sigma Code: 434086), 3-Bromo-5-Nitrosalicylaldehyde (Sigma Code: 652784), 1′,3′-Dihydro-1′,3′,3′-Trimethyl-6-Nitrospiro[2*H*-1-benzopyran-2,2′-(2*H*)-indole] (SP-Sigma) (Sigma Code: 273619), 2-Butanone (MEK) (Sigma Code: 110264), Piperidine (Sigma Code: 411027), Ethanol (Sigma Code: 277649-1l), Methanol (Sigma Code:179337), Methanol-$^{12}C,d_4$ (Sigma Code: 296775), Potassium Bromide (Sigma Code: 221864), HEPES (Sigma Code: H3375), Chloroform (Sigma Code: 437581), Ethyl acetate (Sigma Code: 319902), Tetrahydrofuran (THF) (Sigma Code: 360589), Acetone (Sigma Code: 179124), Acetonitrile (MeCN) (Sigma Code: 360457), 2-Propanol (Isopropanol) (IPA) (Sigma Code: 190764), Phosphate Buffered Saline (Sigma Code: P4417), MES Sodium Salt (Sigma Code: M5057-100g), HEPES Buffer Salts (Sigma Code: H7523). Whatman® qualitative filter paper, Grade 4 (Sigma Code: Z240516), Silica gel on TLC-plate (Sigma Code: 60768) were also obtained from Sigma Aldrich UK. Toluene (Fischer Code: T/2306/15) was obtained from Fischer Scientific, UK. LEDs components were acquired from Roithner Laser Technik, Austria; White LEDs, Based on GaN 27-33 Cd (Roithner Laser Technik Code: 5w4hca-H20-Ultra), UV LEDs (Roithner Laser Technik Code: NS360L-5RLO). IR, UV-Vis and ^{1}H NMR spectra were recorded on an Avatar 370 E.S.P. FT-IR Spectrometer System (Thermo Nicolet), UV-Vis Spectrophotometer UV-2100 (Shimadzu), and FT-NMR System (JNM-ECX Series - Delta V4.3) (Jeol) respectively.

2.3 Synthesis of Carboxylated Spiropyran Dyes

The method applied here is a duplication of Kirkham (1996) and Sesay (2003), which is an adaptation of the method developed by Aizawa *et al.*, (1977). With regards to the method for synthesising the SP-COOH derivatives (SP-COOH-Me and SP-COOH-Br): a further adaptation to Kirkham (1996) and Sesay (2003) was applied. Figure 2.1 shows the reaction scheme for the synthesis of spiropyran dyes using 2,3,3,trimethylindolenine and iodopropionic acid as starting materials, and adding 5-nitrosalicylaldehyde in the presence of piperidine to syntheisis SP-COOH.

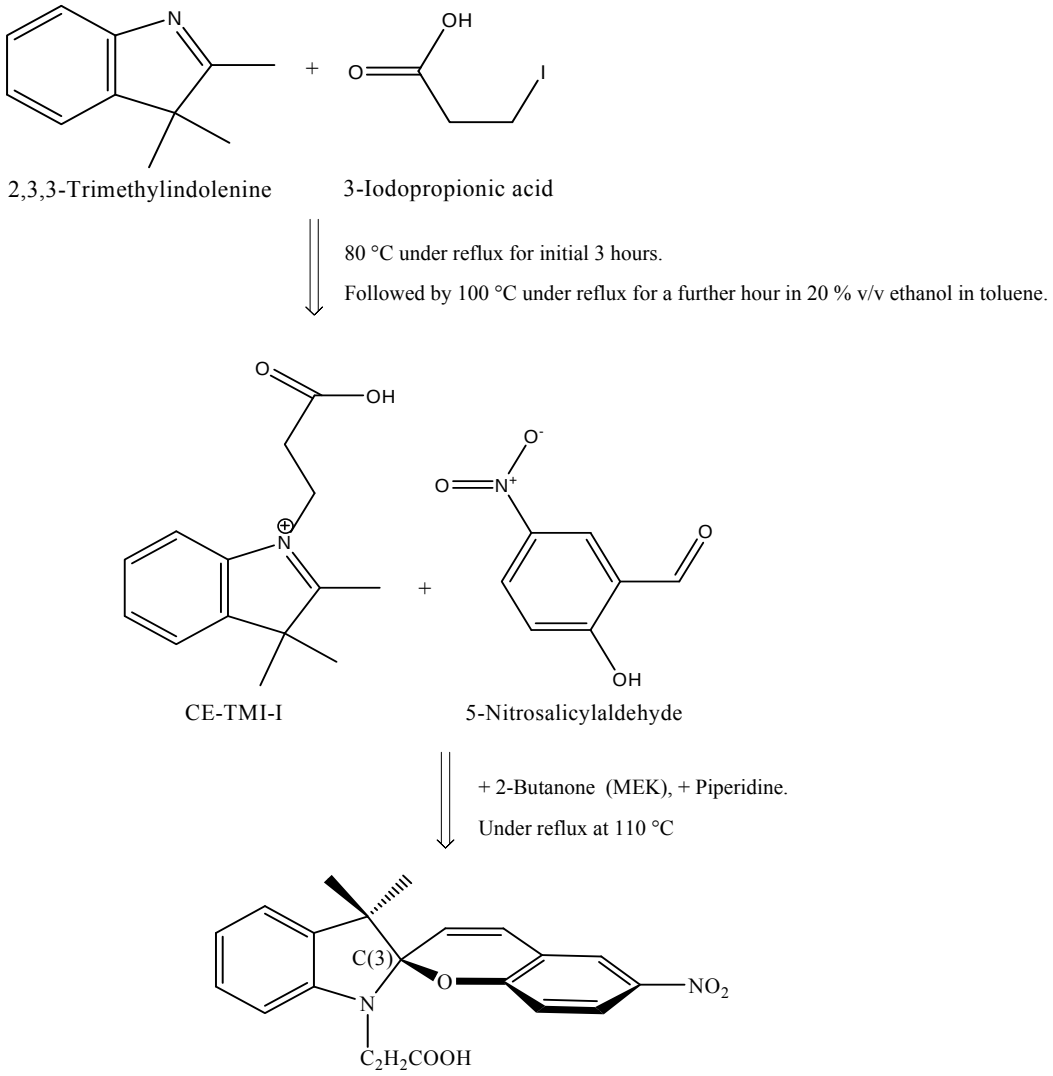


Figure 2.1 : Schematic Reaction Sequence of SP-COOH Synthesis.

2.3.1 Synthesis of l-(2-carboxyethyl)-2,3,3-trimethylindolenium iodide (CE-TMI-I)

An equimolar mixture of 3-Iodopropionic acid (7.55 g) and 2,3,3-Trimethylindolenine (6.00 ml) was heated at 80°C under reflux in a round bottom flask for 3 hours.
93 ml of 20% v/v ethanol in toluene was added to the mixture. The resulting solution was heated at 100°C under reflux for a further hour and then taken off the heat to cool to room temperature. The resulting product was left over night (~ 12 hours) at 4 °C to re-crystallise. The filtrand, a purple crystalline precipitate, was collected by vacuum filtration, and the solution (filtrate) was retained and stored at 4 °C for a further re-crystallisation. This was to make sure there was high yield of product at each stage.
The purple crystals collected were crushed using a glass pestle and mortar then washed with 5% ethanol in toluene under vacuum filtration. (The subsequent filtrate was also retained for further crystallisation at 4 ºC over night and washed with 5% ethanol in toluene to ensure high yield).

In order to attain a pure substance, the washed off purple crystals obtained were refluxed at 100 °C in fresh toluene, using sufficient ethanol to dissolve all the material (approx. 5-10%). This was left overnight at 4 °C to re-crystallise. The crystals were filtered off under vacuum and dried to give a yellow crystalline product. The solid (yellow crystalline) product was again heated under reflux in toluene at 100 °C. Ethanol was added drop wise until the solid was fully dissolved. The solution was then taken off the heat to cool at room temperature, and then left over night at 4 °C to re-crystallise. The resultant white crystalline solid (CE-TMI-I) was filtered, dried and stored in the dark at 4 °C.

2.3.2 Synthesis of Carboxylated Spiropyran Dye (SP-COOH)

The resultant white crystalline solid CE-TMI-I from Section 2.31 was suspended in 2-Butanone (MEK) (500 mg of solid in 600 µl of MEK) in a round bottom flask. To aid solubility, piperidine (125 µl) was added to the suspension, allowed to dissolve and heated under reflux at 110 °C until all solid was in solution (~ 5 minutes).

The reaction mixture was taken off the heat and allowed to cool at room temperature. 250 mg of 5-Nitrosalicylaldehyde was dissolved in 2 ml of MEK and the mixture was added to the reaction mixture in the round bottom flask. The mixture was then reheated under reflux at 110 – 120 °C for 5 minutes. The reaction mixture was left overnight at room temperature to allow the carboxylated spiropyran dye to precipitate. The precipitate (SP-COOH) was filtered and washed under a vacuum with 50 ml of deionised water and left to dry. The resultant reddish solid (SP-COOH) 380.36 mwt. (based on proposed chemical formula) was stored in the dark at 4 °C ready for use. The percentage product yield was 67%.

2.3.3 Synthesis of 8'-Methoxy Derivative of SP-COOH (SP-COOH-Me)

From Section 2.3.1 the resultant white crystalline solid CE-TMI-I was suspended in 2-Butanone (MEK), dissolved by the addition of piperidine and heated under reflux at 110 °C until all solid was in solution. However the following step which involved 5-Nitrosalicylaldehyde component was substituted with 3-Methoxy-5-Nitrosalicylaldehyde and the method was just as in section 2.3.2. The resultant solid (SP-COOH-Me), dark red in colour, molecular weight of 411 (based on proposed chemical formula) was stored in the dark at 4 °C ready for use. The percentage product yield was 59%. Figure 2.2 shows the reaction scheme for the synthesis of SP-COOH-Me using 2,3,3,trimethylindolenine and iodopropionic acid as starting materials and adding 3-methoxy-5-nitrosalicylaldehyde in the presence of piperidine to syntheisis SP-COOH-Me.

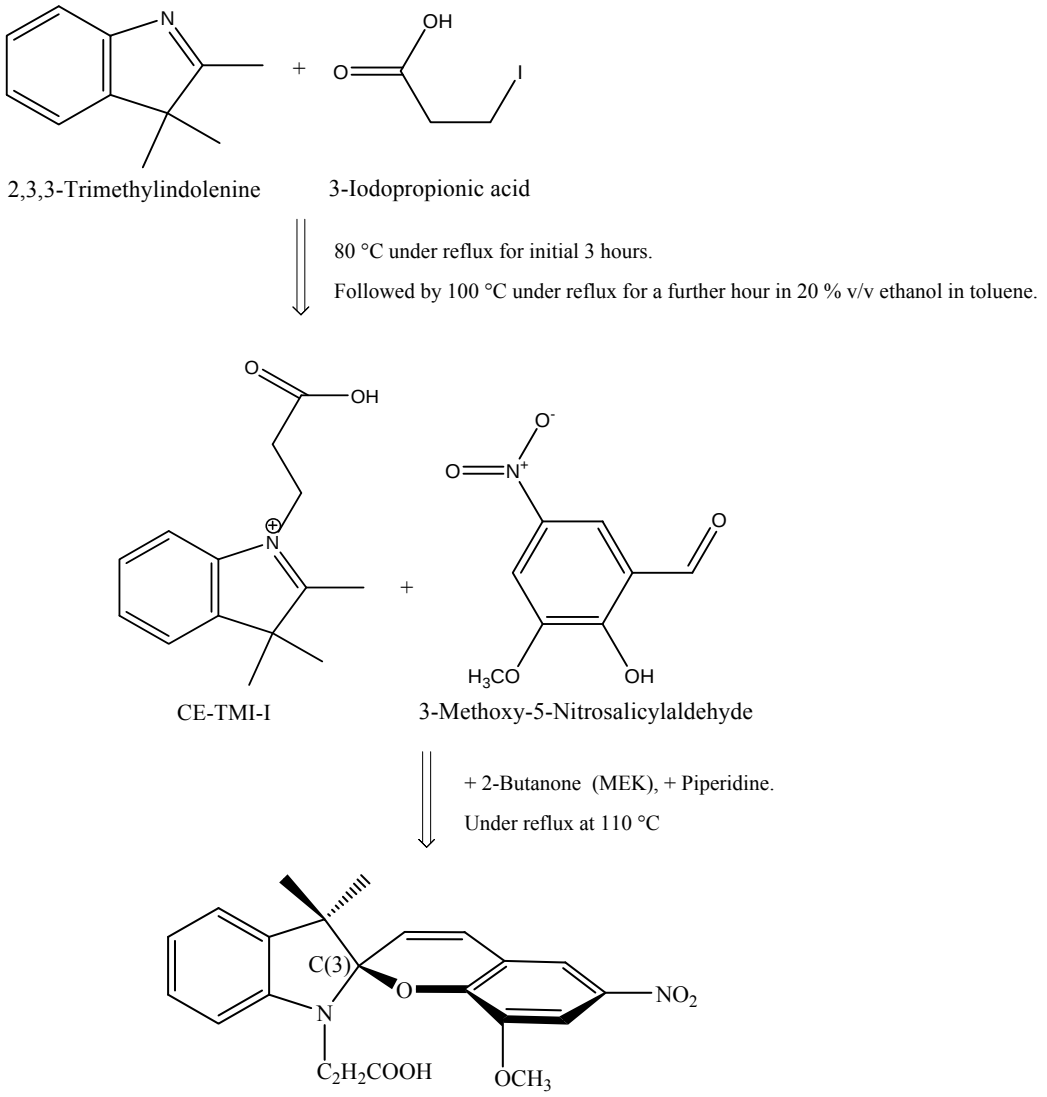


Figure 2.2 : Schematic Reaction Sequence of SP-COOH-Me Synthesis.

2.3.4 Synthesis of 8'-Bromo Derivative of SP-COOH (SP-COOH-Br)

The synthesis of SP-COOH-Br, was conducted as in Section 2.3.2 however 3-Bromo-5-Nitrosalicylaldehyde was used in place of 5-Nitrosalicylaldehyde. A dark brown solid, molecular weight of 460 (based on proposed chemical formula) was collected. Product percentage yield of 64%. Figure 2.3 shows the reaction scheme for the synthesis of SP-COOH-Br using 2,3,3,trimethylindolenine and iodopropionic acid as starting materials and adding 3-bromo-5-nitrosalicylaldehyde in the presence of piperidine to syntheisis SP-COOH-Br.

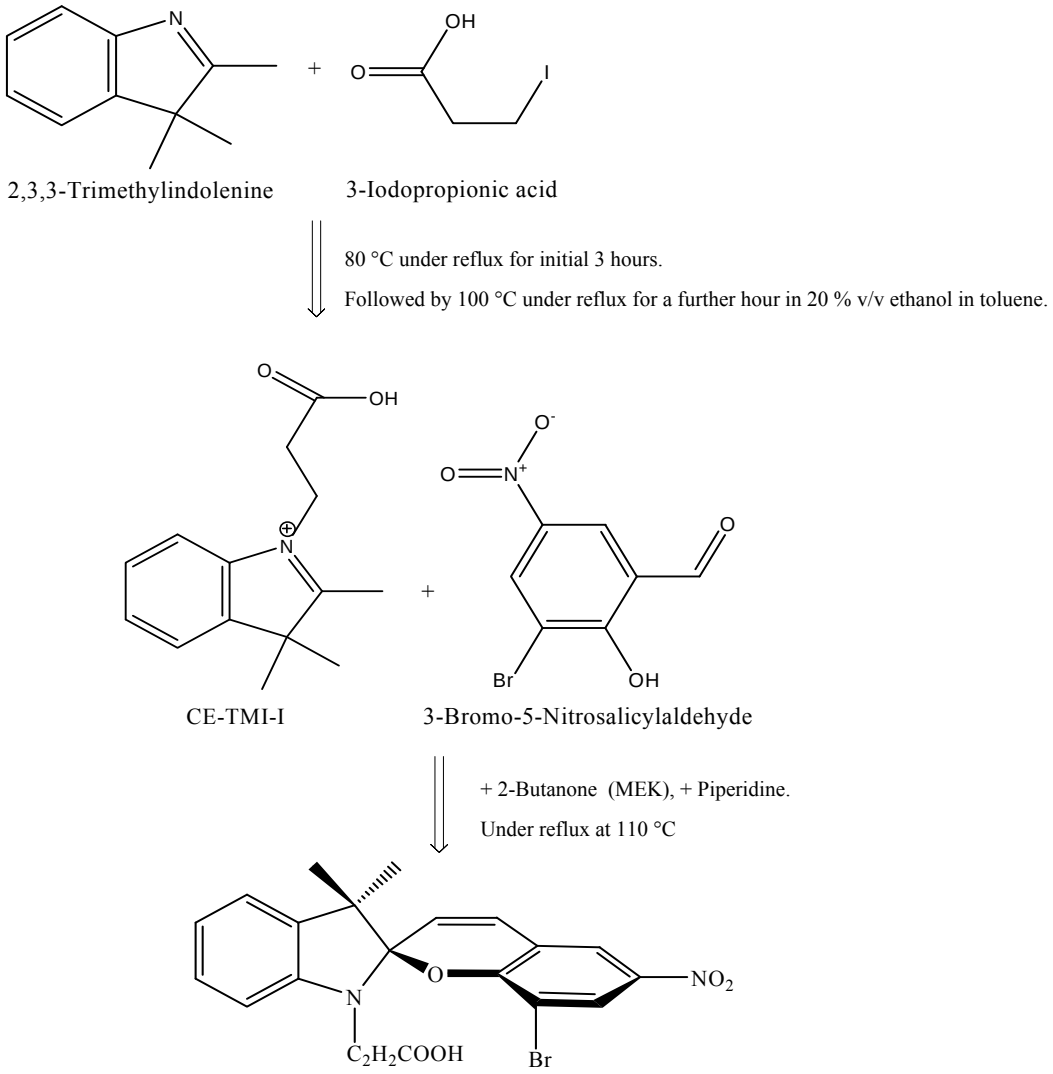


Figure 2.3 : Schematic Reaction Sequence of SP-COOH-Br Synthesis.

2.4 Characterisation of Synthesised Spiropyran Dyes

2.4.1 Introduction

The confirmation of the identity of the synthesised product is essential prior to further application or/and analysis. Three analysis methods namely Thin Layer Chromatography (TLC), Nuclear Magnetic Resonance Spectroscopy (NMR) and Infrared Spectroscopy (IR) are used in this study. Spiropyrans are known to be soluble in ethanol, methanol and other similar organic solvents, hence during characterisation, these solvents were used where applicable. However the effect for solvent choice and other environmental conditions on the photoswitching properties of the dyes are further studied in subsequent sections within this chapter.

2.4.2 Thin Layer Chromatography Analysis

TLC is known to be a simple, quick, and inexpensive procedure that gives information on how many components are in a mixture. This technique can also give information on the relative pureness of a compound. To determine the successful synthesis of the carboxylated dyes, the reaction components, the end products and control (SP-Sigma) were analysed.

TLC was observed using silica plates and aluminium oxide plate using 99.9% ethanol as the mobile liquid phase to determine the relative purity. A solution of the reaction components, the end products and control were dissolved in 100 % ethanol and 25 µl spotted onto the TLC plates. All of the reaction compounds used for the dye synthesis was spotted at concentration of 1 mg/ml. The resulting positions of compounds were viewed in ambient light (*i.e.* silica oxide plate) and under UV light (245 nm) (for aluminium oxide plates) and the positions measured. The R_f values were determined as the distance of the compound leading edge divided by the solvent front distance. Table 2.2 shows the R_f values.

Table 2.2 : TLC Mean R_f Values of Dye Synthesis Components and Products.

R_f value measurements of the reactant components for the synthesis of the carboxylated spiropyran dyes.

Plate Key	Component Name	Mean R_f Value
A	3-Iodopropionic Acid	0.39; 0.87
B	2,3,3-Trimethylindolenine	0.94
C	CE-TMI-I	0.48; 0.78
D	5-Nitrosalicylaldehyde	0.94
E	3-Bromo-5-Nitrosalicylaldehyde	0.94
F	3-Methoxy-5-Nitrosalicylaldehyde	0.94
G	SP-COOH	0.80
H	SP-COOH-Me	0.76
I	SP-COOH-Br	0.85
J	SP-Sigma	0.79

With regards to purity testing via TLC using silica plates, the most polar compounds are expected to have a stronger interaction with the silica plate; therefore they will be more capable to dispel the mobile phase from the binding places. Consequently, the less polar compounds are expected to move higher up the plate (resulting in a higher R_f value). Within Table 2.2 R_f values for 3-Methoxy-5-Nitrosalicylaldehyde, 3-Bromo-5-Nitrosalicylaldehyde, 5-Nitrosalicylaldehyde and 2,3,3-Trimethylindolenine were 0.94 which suggests that these components rapidily moved through the mobile liquid phase. However, CE-TMI-I had considerably different (R_f 0.48 and 0.78) profile values than the either 3-Iodopropionic Acid (R_f 0.39 and 0.87) which migrated along with the solvent line and 2,3,3-Trimethylindolenine (R_f 0.94). The band at R_f 0.48 is believed to be CE-TMI-I, with the band at R_f 0.78 being impurities in the product. The spiropyran dyes product SP-COOH, SP-COOH-Me, SP-COOH-Br, SP-Sigma produced a clear single band at R_f 0.80, 0.76, 0.85, 0.79 respectively, which were highly coloured when illuminated in UV and faded quickly when exposed to visible light. Comparing the R_f values of the synthesised dyes with SP-Sigma, it can be concluded that the synthesised dyes were pure. The sizes of the spots were also

used to confirm this. These results give an initial indication of successful synthesis of dye.

2.4.3 Nuclear Magnetic Resonance Spectroscopy Analysis

NMR spectroscopy is one of the principal techniques used to obtain chemical, electronic and structural information about molecules. ^{1}H NMR spectroscopy was observed for the synthesised dyes and SP-Sigma with deuterated methanol as solvent choice; deuterated acetonitrile was initially used but methanol gave better defined spectrum peaks. 10 mg of the test sample was dissolved in deuterated acetonitrile (~ 600 µl) and filtered (using a glass pasteur pipette, tip covered with a thin layer of tissue paper as the filter). The filtered sample was then transferred into an NMR tube to a depth of ~ 5 cm (which is equivalent to ~ 600 µl of solution). Below is a summary of the results (JACS format) of the ^{1}H NMR spectra (graph available in Appendix).

(SP-Sigma) ^{1}H NMR (400 MHz, ACETONITRILE-D3) δ 8.11 – 7.92 (m, 2H), 7.21 – 6.98 (m, 3H), 6.88 – 6.76 (m, 1H), 6.71 (dd, *J* = 0.5, 9.0, 1H), 6.57 (d, *J* = 7.8, 1H), 5.92 (d, *J* = 10.4, 1H), 2.70 (s, 3H), 2.13 (s, 6H), 1.92 (dt, *J* = 2.5, 4.9, 3H), 1.24 (s, 3H), 1.18 – 1.05 (m, 3H).

(SP-COOH) ^{1}H NMR (400 MHz, ACETONITRILE-D3) δ 8.09 – 7.90 (m, 16H), 7.22 – 6.96 (m, 25H), 6.80 – 6.65 (m, 17H), 6.57 (d, *J* = 7.7, 9H), 5.93 (d, *J* = 10.4, 9H), 3.18 – 3.10 (m, 1H), 2.93 – 2.85 (m, 1H), 2.72 (s, 24H), 2.49 – 2.51 (m, 1H), 2.04 – 1.84 (m, 70H), 1.19 (t, *J* = 30.0, 51H), 1.04 – 0.96 (m, 1H).

(SP-COOH-Me) ^{1}H NMR (400 MHz, ACETONITRILE-D3) δ 8.09 – 7.90 (m, 16H), 7.22 – 6.96 (m, 25H), 6.89 – 6.65 (m, 17H), 6.57 (d, *J* = 7.7, 9H), 5.93 (d, *J* = 10.5, 9H), 3.18 – 3.10 (m, 1H), 2.93 – 2.85 (m, 2H), 2.72 (s, 24H), 2.79 – 2.51 (m, 1H), 2.04 – 1.84 (m, 70H), 1.19 (t, *J* = 30.0, 51H), 1.04 – 0.96 (m, 1H).

(SP-COOH-Br) ^{1}H NMR (400 MHz, ACETONITRILE-D3) δ 7.12 (d, *J* = 21.6, 2H), 7.02 (d, *J* = 10.4, 1H), 6.84 (s, 1H), 6.67 (dd, *J* = 8.4, 28.1, 2H), 5.95 (d, *J* = 10.4, 1H), 2.56 – 2.48 (m, 1H), 1.91 (dt, *J* = 2.5, 4.9, 14H), 1.25 (s, 2H), 1.13 (s, 2H).

The NMR data accounted most ^{1}H, however ^{1}H of the synthesised dye for the carbon chain linking COOH to the entire molecule was not accounted for. This was however repeated with deuterated methanol but was still no spectral data for the proton. The next logical step was to conduct the functional group analysis via IR spectroscopy.

2.4.4 Infrared Spectroscopy Analysis

Following the characterisation via TLC and NMR analysis, IR spectroscopy was used to analyse the presence for the carboxyl (functional) group attached to the compounds synthesised. 1 mg solid dye was first crushed in a marble mortar with a pestle. 60 mg of fine potassium bromide (to remove scattering effects from large crystals) was added and the mixture was mechanical pressed at 10 tons to form a translucent pellet through which the absorption spectra were observed. As a control SP-Sigma IR analysis was compared to the results attained, since this compound (SP-Sigma) is not expected show any indication of a carboxyl group.

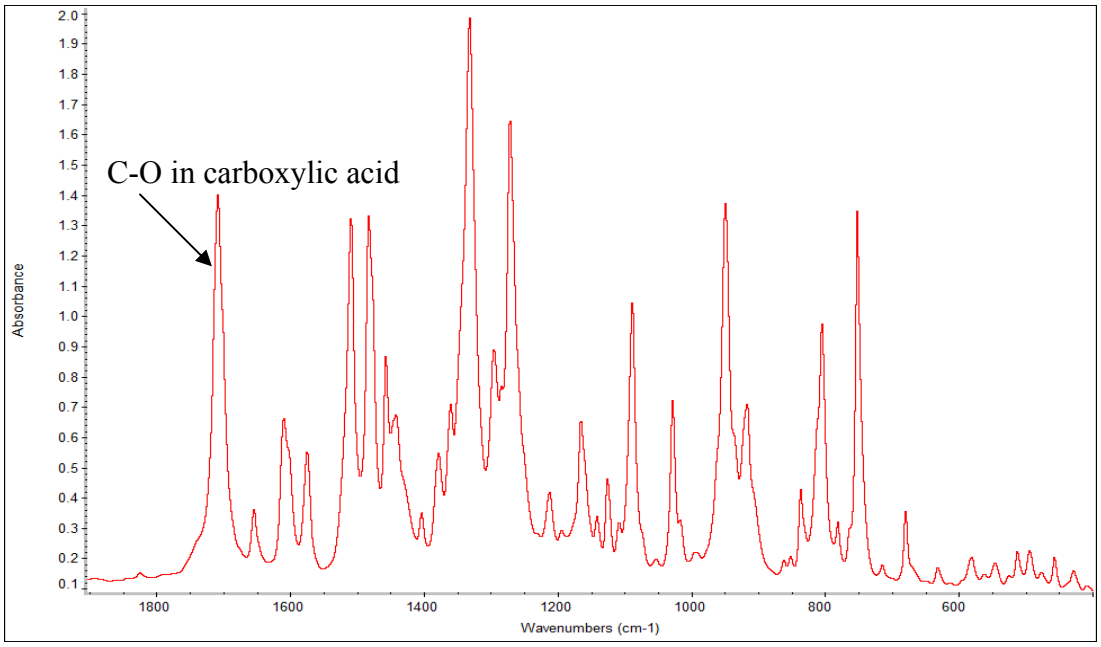


Figure 2.4 : IR Spectrum of SP-COOH.

The carboxyl functional group is indicated on the graph between 1690 and 1750 wavenumber (cm^{-1}).

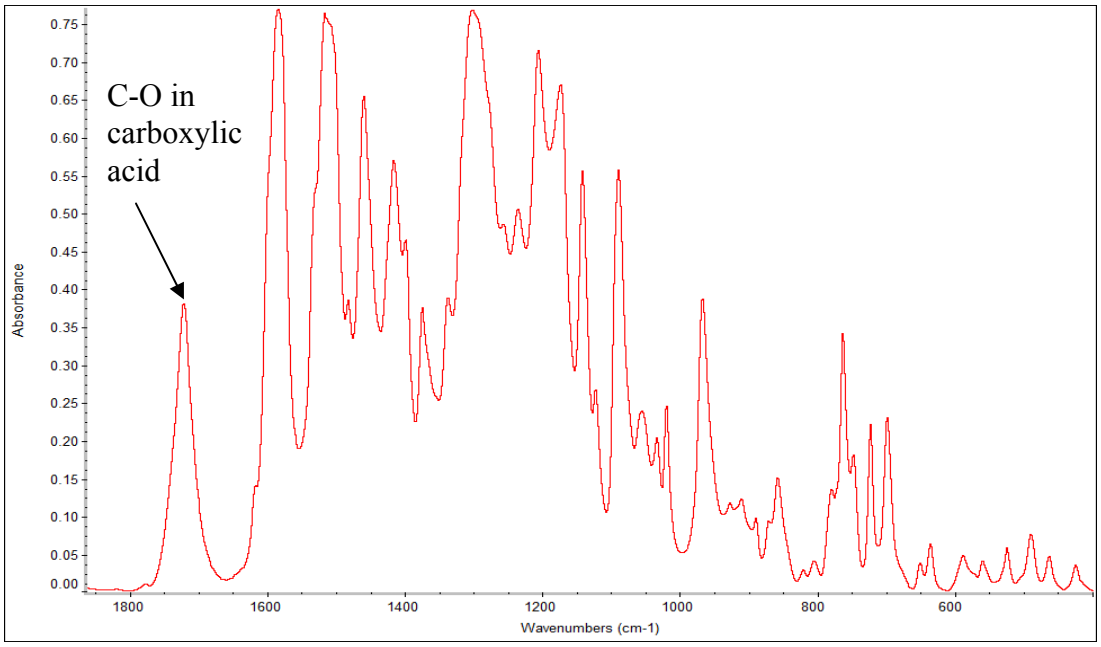


Figure 2.5 : IR Spectrum of SP-COOH-Br.

The carboxyl functional group is indicated on the graph between 1690 and 1750 wavenumber (cm^{-1}).

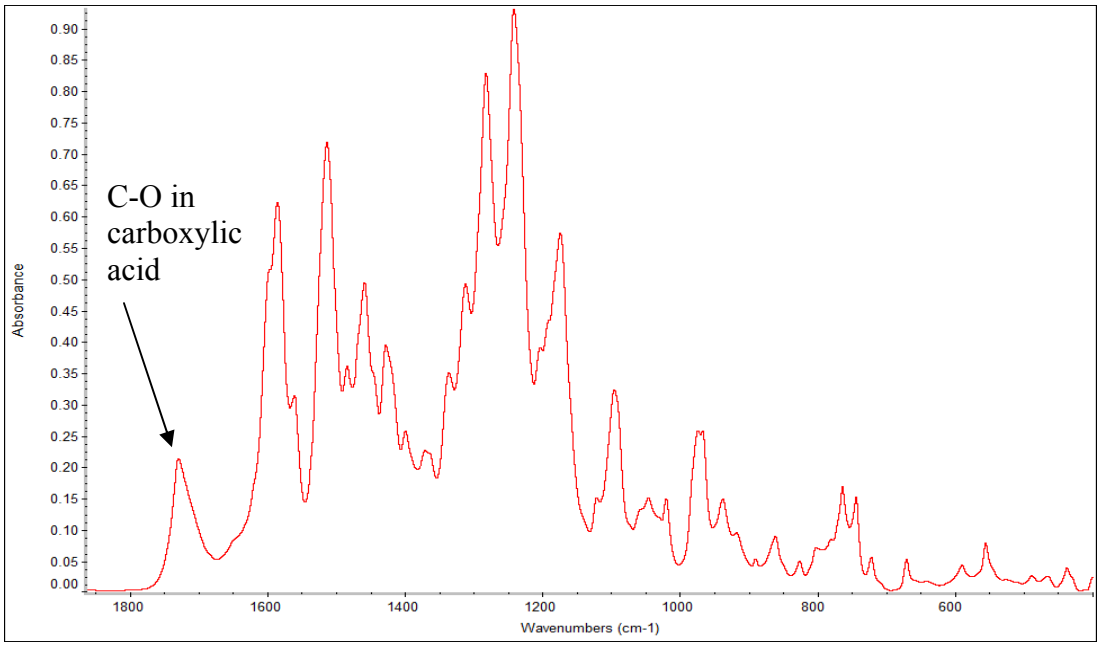


Figure 2.6 : IR Spectrum of SP-COOH-Me.

The carboxyl functional group is indicated on the graph between 1690 and 1750 wavenumber (cm^{-1}).

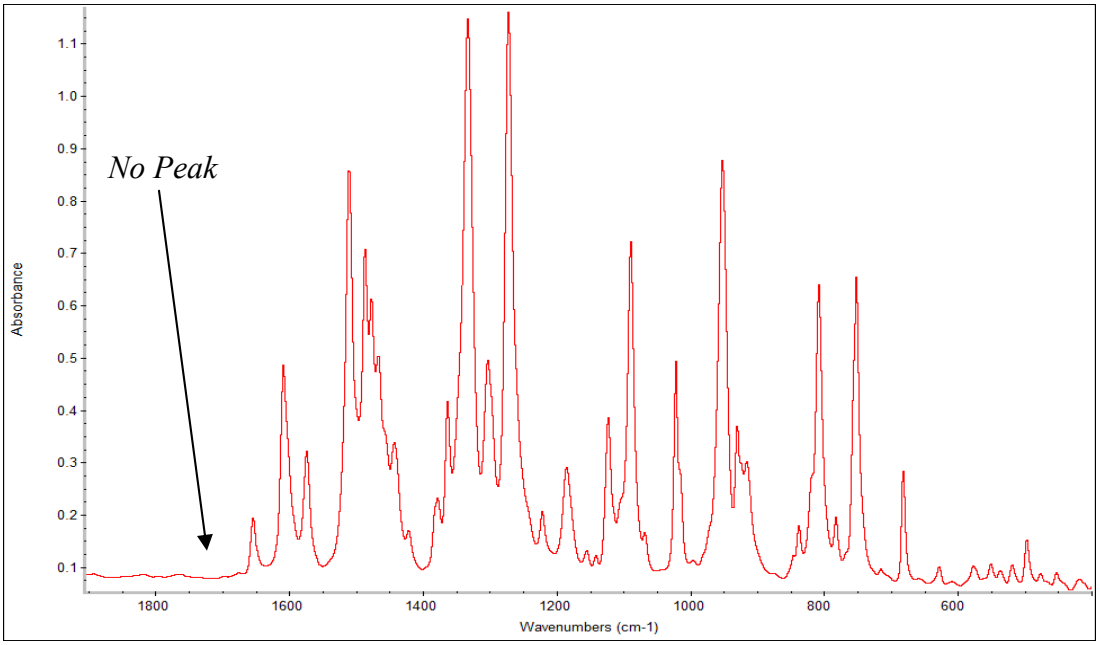


Figure 2.7 : IR Spectrum of SP-Sigma.

No indication of a carboxyl functional group on spectrum between 1650 and 1750 wavenumber (cm^{-1}) as observed with the other synthesised dyes Figures 2.4, 2.5 and 2.6.

Figures 2.4, 2.5 and 2.6 show the IR absorption spectra of SP-COOH, SP-COOH-Br and SPCOOH-Me respectively. An absorption peak at around 1720 cm^{-1} in the spectra is assigned to the C-O in carboxylic acid functional group. In the case of the SP-Sigma (Figure 2.7), as expected, there was no suggestion of the presence of C-O bonds. Following these results, it is evident that each of the synthesised dyes possesses a carboxyl functional group.

2.5 Photoswitching of Synthesised Dyes

2.5.1 Introduction

Reversible, photo induced, colour change of spiropyrans in solution were first described in 1926 (Tinland *et al.*, 1973). When spiropyrans dissolved in ethanol are exposed to different wavelenghts of light (visible light and UV light) or placed in the dark, photochromism occurs which involves the two photo isomers, the neutral closed spiro form and the zwitterionic opened merocyanine form. This change is characterised by large differences in geometry and polarity. The formation of the merocyanine form involves the C–O bond cleavage, resulting in the molecule becoming metastable and amphoteric as explained in section 1.3.2. Due to the change in polarity of the isomers during photoswitching, the polarity of the environment (i.e. the solvent dissolved in) is a pivotal component in the switching capabilities of the dyes. However to display the photoswitching competence of the synthesised dyes, ethanol was used (as it is the most frequently used solvent in spiropyran dyes photoswitching).

It was ideal to also set up the form of illumination to be implemented through out this study since light source is the activators of photochromism. To date, literature reports on spiropyrans switching has involved the use of high power light sources such as mercury arc lamps and lasers. The high powered light source sometimes emits heat energy which is crucial to the fatigue phenomenon and thermochromism (later looked into in a subsequent section). The aim is to reduce any controllable limiting factors (such as heat energy) in this study and as such the use of Light Emitting Diodes (LEDs) as light sources would accomplish that. Stitzel *et al*., 2006 and Radu *et al*., 2007 have also shown the use of LEDs as light sources in the photoswitching of photochromic dyes. LED sources require significantly less power, emit less heat and are inexpensive alternatively to other light sources such as the arc lamps.

2.5.2 Photochromic Activity

To demonstrate the photochromic activity of the synthesised dyes, two experiments were performed. The first involved the photoswitching of dyes under UV and visible light. The second involved the dark adaptation switching of the spiropyran form to the coloured merocyanine form as carboxylated spiropyrans are known to exhibit negative (or reverse) photochromism as explained in Chapter 1.

0.25 μg/ml of the synthesised dyes and the control dye were prepared in ethanol at 20 °C. The solution was initially exposed to the white LED (27-33 cd @ 30mA) which triggered the conversion to the spiropyran (colourless, closed) form within 30 seconds. 1 ml was placed in a quartz cuvette (path length: 1 cm). With appropriate solvent (ethanol) as the blank, the dye solutions absorption spectra were observed between 400 and 700 nm using a UV-Vis Spectrophotometer. With the solution still located in the UV-Vis Spectrophotometer, UV illumination via a UV LED (360nm 1.2 -1.8 mW @ 20mA) was exposed to the solution for 10 minutes (with minimum ambient visible light present), the absorption spectra were observed between 400 and 700 nm. To ensure the dye reverted back to the spiropyran form, the solution was exposed to white LED lights and the absorption spectra were observed between 400 and 700 nm.

The dark adaptation experiment was observed. This was conducted by ensuring the dye was in the spiropyran form by initial exposure to white LED light, and observing the absorption spectra in the UV-Vis Spectrophotometer (dark environment) every 15 minutes for 2 hours.

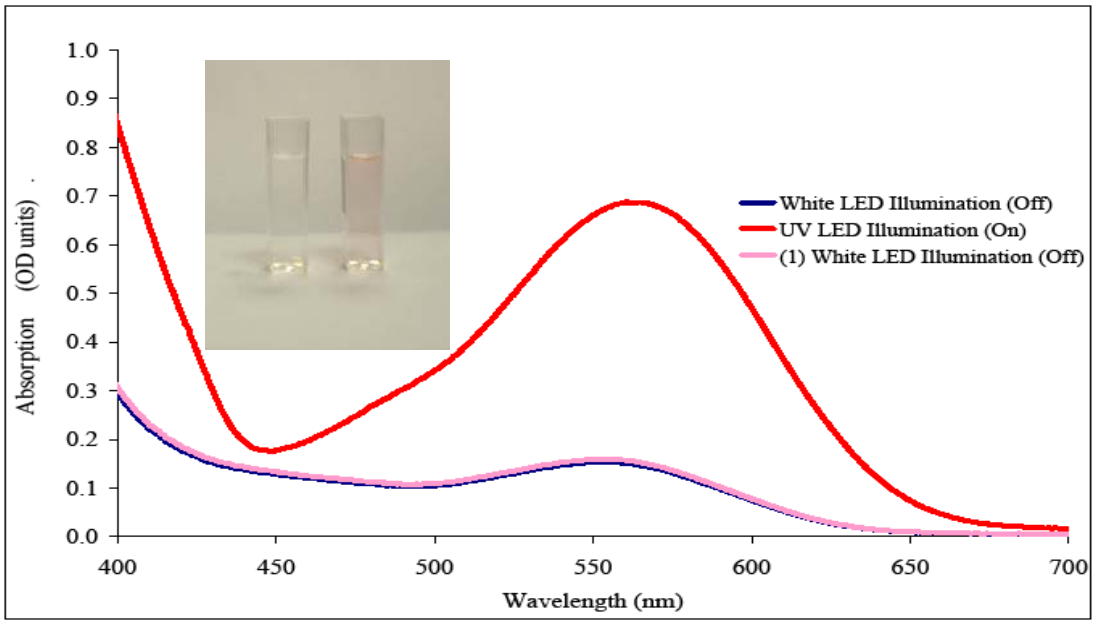


Figure 2.8 : UV/Vis Absorption Spectrum of SP-COOH in Ethanol.

Sample contained 0.25 µg/ml (6.6 x 10^{-6} M) of dye in ethanol at 20 °C. Illumination was conducted with the use of UV LED (360nm) for 10 minutes and white LED for 30 seconds.

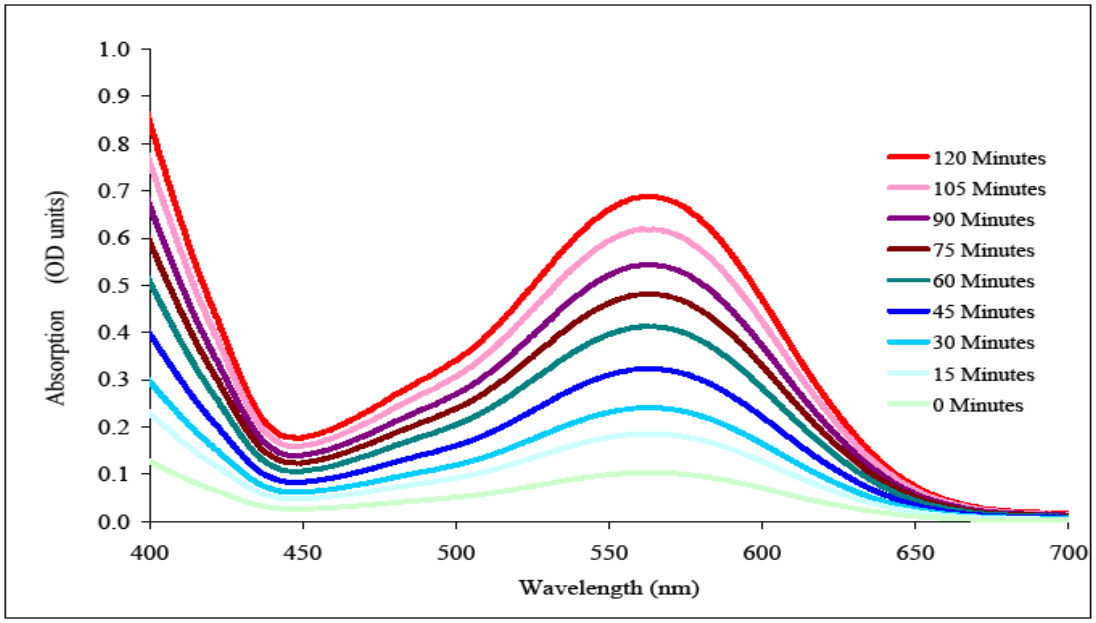


Figure 2.9 : Dark Adaptation Photoswitching of SP-COOH.

Photoswitching of the spiropyran form to the merocyanine form. Sample contained 0.25 µg/ml (6.6 x 10^{-6} M) of dye in ethanol. Initial illumination with white LED for 30 seconds, then placed in the UV-Vis Spectrophotometer (dark environment), and scanned every 15 minutes for 2 hours at 20 °C.

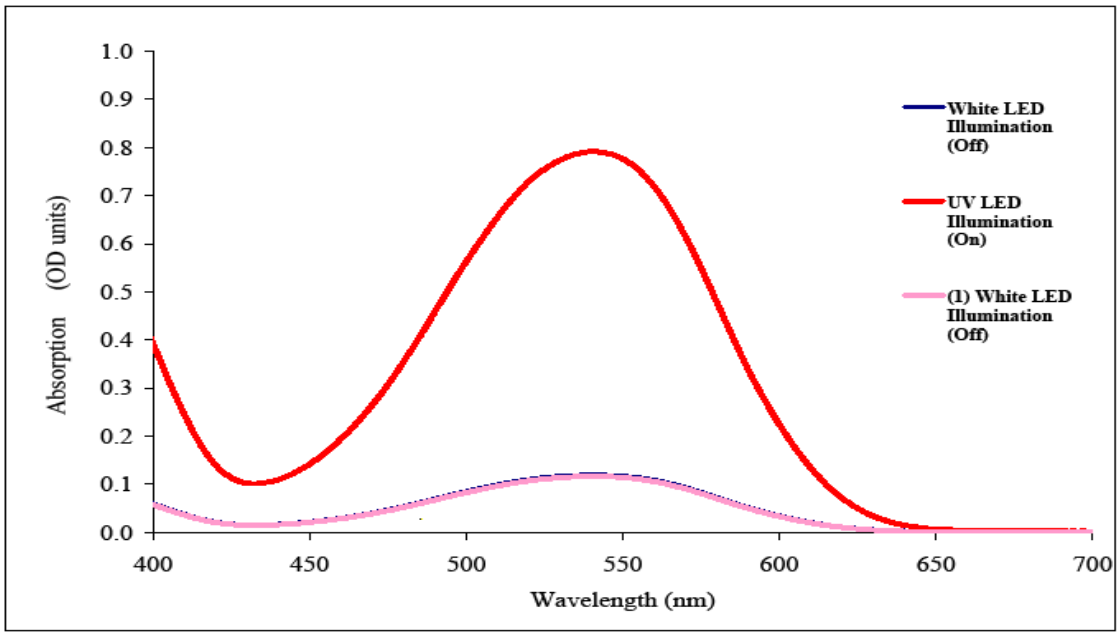


Figure 2.10 : UV/Vis Absorption Spectrum of SP-COOH-Br in Ethanol.

Sample contained 0.25 µg/ml (6.1 x 10^{-6} M) of dye in ethanol at 20 °C. Illumination was conducted with the use of UV LED (360nm) for 10 minutes and white LED for 30 seconds.

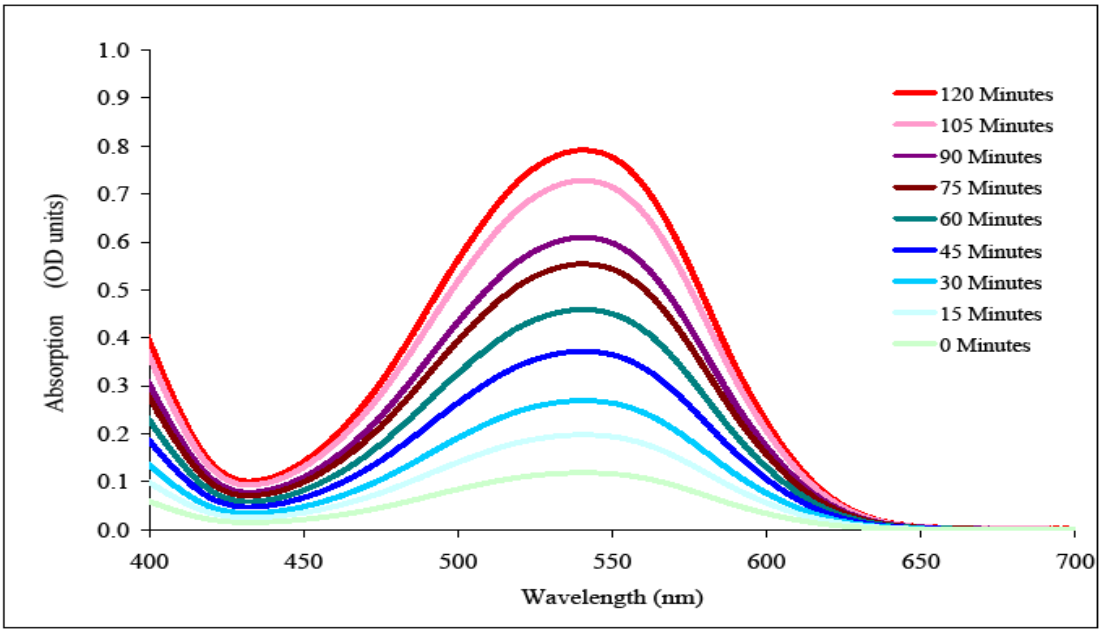


Figure 2.11 : Dark Adaptation Photoswitching of SP-COOH-Br.

Photoswitching of the spiropyran form to the merocyanine form. Sample contained 0.25 µg/ml (6.1 x 10^{-6} M) of dye in ethanol. Initial illumination with white LED for 30 seconds, then placed in the UV-Vis Spectrophotometer (dark environment), and scanned every 15 minutes for 2 hours at 20 °C.

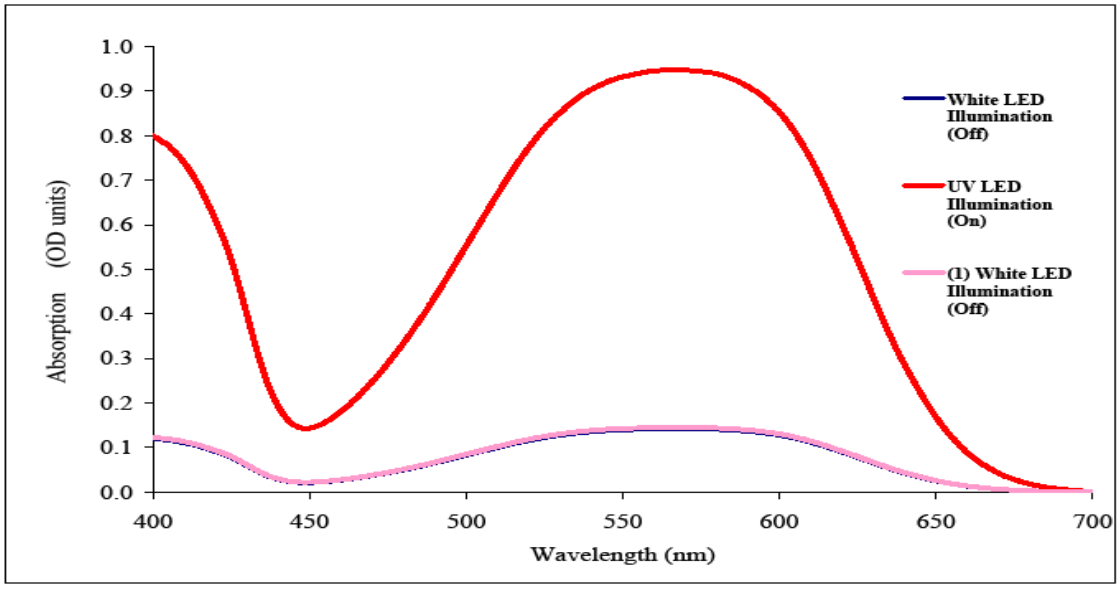


Figure 2.12 : UV/Vis Absorption Spectrum of SP-COOH-Me in Ethanol.

Sample contained 0.25 µg/ml (6.2 x 10^{-6} M) of dye in ethanol at 20 °C. Illumination was conducted with the use of UV LED (360nm) for 10 minutes and white LED for 30 seconds.

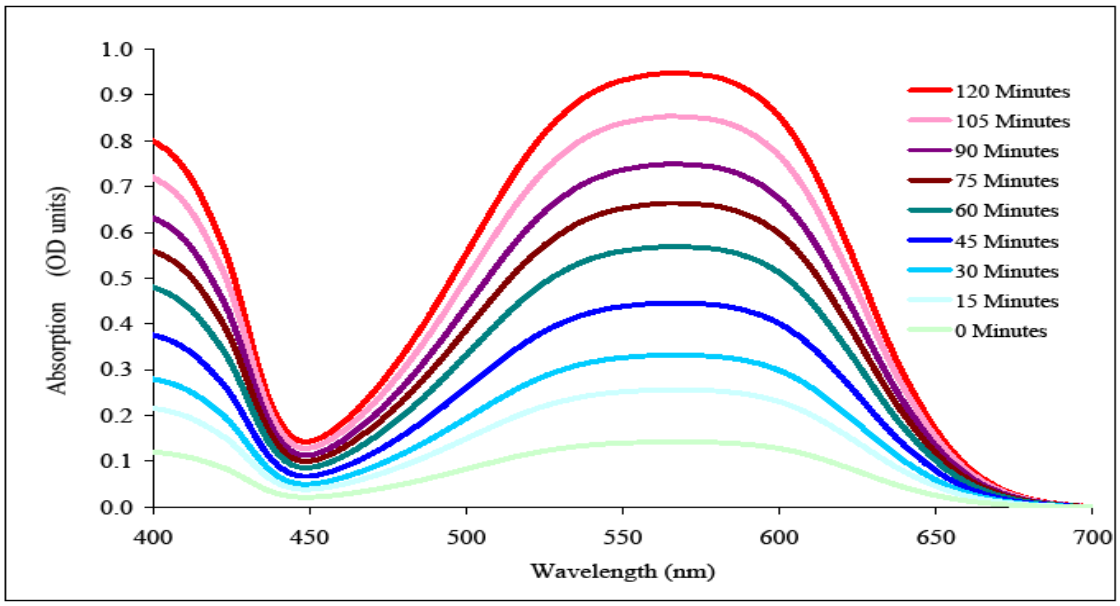


Figure 2.13 : Dark Adaptation Photoswitching of SP-COOH-Me.

Photoswitching of the spiropyran form to the merocyanine form. Sample contained 0.25 µg/ml (6.2 x 10^{-6} M) of dye in ethanol. Initial illumination with white LED for 30 seconds, then placed in the UV-Vis Spectrophotometer (dark environment), and scanned every 15 minutes for 2 hours at 20 °C

Figures 2.8 to 2.13 confirmed that all three synthesised dyes (SP-COOH, SP-COOH-Br and SPCOOH-Me) were photochromic and could readily be inter converted between isomers with LEDs.

Spiropyran compounds are known to exhibit normal photochromism, which implies that they are colourless to pale yellow solutions becoming highly coloured when irradiated with UV light and then reverting back to the colourless spiropyran form upon irradiation with visible light or via dark adaptation when left in the dark. However the presence of the carboxylic group of the dye makes them exhibit negative photochromism which means the thermodynamically stable form is the merocyanine form, hence the dye are still colourless to pale yellow solutions becoming highly coloured when irradiated with UV light and then reverting back to the colourless spiropyran form upon irradiation with visible light, but during dark adaptation the dye exhibit the coloured merocyanine form. All three synthesised dyes exhibited negative photochromism as the merocyanine form was evident in the dark and the inter conversion rate from spiropyran to merocyanine in the dark was slow but feasible. The variation in spectra peaks (absorption maxima) was the main observable difference between the dyes. This was accounted for by the bromine and methoxy substituent although this did not interfere with photoswitching.

2.6 Solvent Effect on Synthesised Spiropyran Dyes

2.6.1 Introduction

Spiropyrans dyes are known to also under-go solvatochromism which is the variation of the electronic spectroscopic properties (absorption) of a chemical species, induced by solvents. In this section the effect of photochromism based on a variety of solvents on the synthesised dyes was observed.

Minkin, (2004) stated that the solvatochromic behaviour of spiropyrans; shows itself as pronounced changes in the position and intensity of their UV/Vis absorption bands. The variation in the polarity of the medium (solvent-dye), relates to the shift of the

equilibrium (between spiropyran and merocyanine form). Solvatochromism is also related to differences in solute-solvent interactions in solvents of different polarity.

The study here is to establish whether the synthesised dyes are subjected to the solvatochromic behaviour. Polar environment would be expected to stabilize the zwitterionic structure of the open merocyanine form and with increasing solvent polarity; the absorption band would be expected to undergo a hypsochromic (or blue) shift. The blue shift is seemingly caused by the extra interaction between polar solvents and ionic merocyanine (Lin, 2003). The synthesised spiropyran dyes were observed in the various solvent shown in Table 2.3

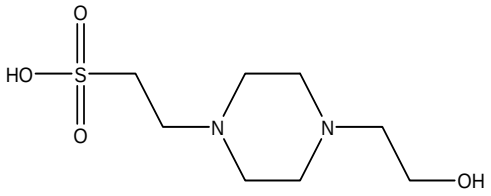
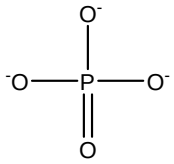
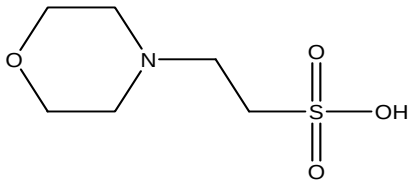
Table 2.3 : Organic Solvents in which Photochromism was Observed

Solvents details include their boiling point and polarity expressed in dielectric constants.

Solvent	Chemical formula	Boiling point	Dielectric constant
Non-Polar Solvents			
Toluene	C_6H_5-CH_3	111 °C	2.4
Chloroform	$CHCl_3$	61 °C	4.8
Ethyl acetate	CH_3-C(=O)-O-CH_2-CH_3	77 °C	6.0
Polar Aprotic Solvents			
Tetrahydrofuran (THF)	CH_2-CH_2-O-CH_2-CH_2	66 °C	7.5
Acetone	CH_3-C(=O)-CH_3	56 °C	21.0
Acetonitrile	CH_3-C≡N	82 °C	37.0
Polar Protic Solvents			
Isopropanol	CH_3-CH(-OH)-CH_3	82 °C	18.0
Ethanol	CH_3-CH_2-OH	79 °C	24.0
Methanol	CH_3-OH	65 °C	33.0

In addition to these solvent, the dyes were also observed in the aqueous buffers shown in Table 2.4, since these will be the applicable environment in which the end use of dye-protein will be observed.

Table 2.4 : Aqueous Buffers in which Photochromism was observed

	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid, 0.1 M **HEPES (Buffer)**
	Phosphate buffered saline, 0.1 M **PBS (Buffer)**
	2-(N-morpholino)ethanesulfonic acid, 0.1 M **MES (Buffer)**

2.6.2 Non Polar Solvents Effect on Dye Photoswitching

0.25 µg/ml of dye was prepared in toluene, chloroform and ethyl acetate at 20 °C. The solutions were treated as in the photochromic activity study, *i.e.* solutions were initially exposed to the white LED (27-33 cd @ 30mA) for 30 seconds. Of this solution 1 ml was placed in a quartz cuvette (path length: 1 cm). With appropriate solvents as blanks, the dye solution was observed between 400 and 700 nm using a UV-Vis Spectrophotometer. The solutions were then exposed to the UV LED (360nm 1.2 -1.8 mW @ 20mA, 5 mm clear epoxy) for 10 minutes and the peak adsorption wavelength were observed. Figure 2.14 displays the relationship between the peak adsorption wavelengths with solvent types.

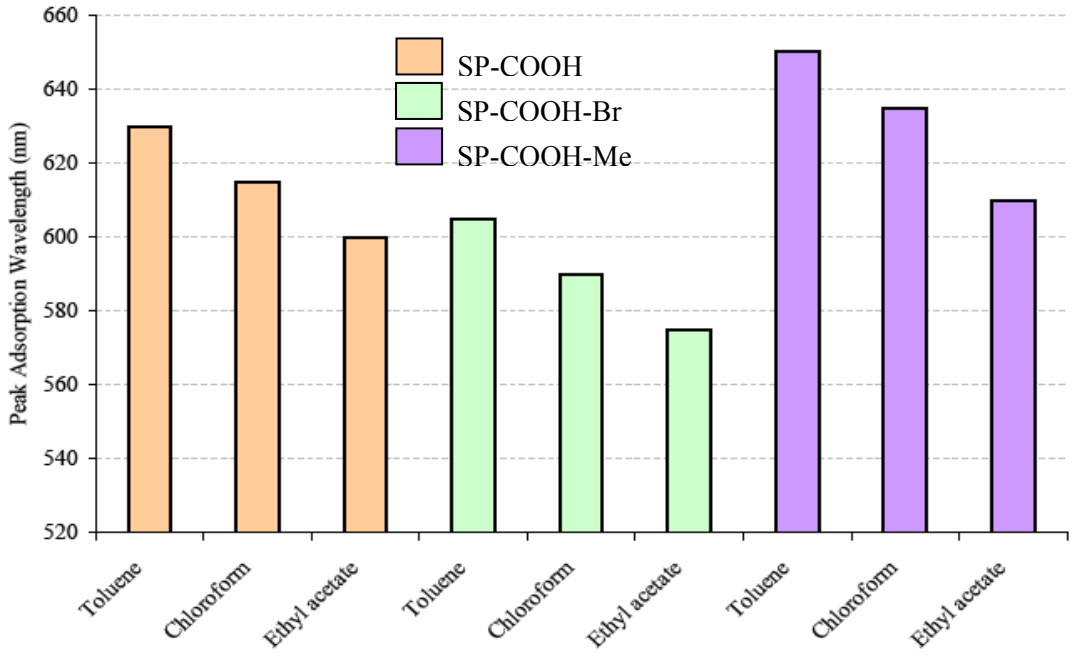


Figure 2.14 : Non Polar Solvents Effect on Dye Photoswitching.

0.25 µg/ml of dye solutions at 20 °C were exposed to visible light and then UV light and the peak adsorption wavelength was noted (under UV illumination).

2.6.3 Polar Protic Solvents Effect on Dye Photoswitching

With regards to polar solvent effect assessment on photochromism of the synthesised dyes, the polar solvents were sub classed into protic and aprotic. Protic solvents were first assessed. Protic solvents generally contain dissociable H^+ (the molecules of such solvents can donate a proton). 0.25 µg/ml of dye was prepared in isopropanol, ethanol and methanol (20 °C). The solutions were treated as in the photochromic activity study, i.e. solutions were initially exposed to the white LED (27-33 cd @ 30mA) for 30 seconds. Of this solution 1 ml was placed in a quartz cuvette (path length: 1 cm). With appropriate solvents as blanks the dye solution was scanned between 400 and 700 nm using a UV-Vis Spectrophotometer. The solutions were then exposed to the UV LED (360nm 1.2 -1.8 mW @ 20mA) for 10 minutes and the peak adsorption wavelengths were observed. Figure 2.15 displays the relationship between the peak adsorption wavelengths with solvent types.

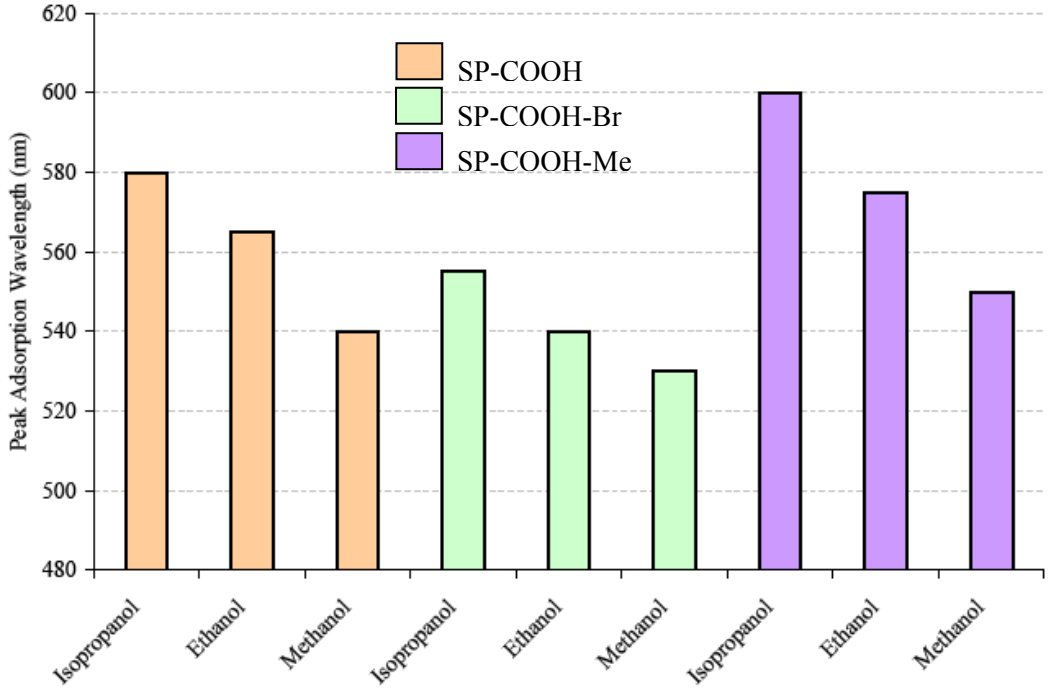


Figure 2.15 : Polar Protic Solvents Effect on Dye Photoswitching.

0.25 µg/ml of dye solutions at 20 °C were exposed to visible light and then UV light and the peak adsorption wavelength was noted (under UV illumination).

2.6.4 Polar Aprotic Solvents Effect on Dye Photoswitching

0.25 µg/ml of dye was prepared in tetrahydrofuran, acetone and acetonitrile (20 °C). The solutions were treated as in the photochromic activity study, *i.e.* solutions were initially exposed to the white LED (27-33 cd @ 30mA,) for 30 seconds. Of this solution 1 ml was placed in a quartz cuvette (path length: 1 cm). With appropriate solvents as blanks the dye solution was scanned between 400 and 700 nm using a UV-Vis Spectrophotometer. The solutions were then exposed to the UV LED (360nm 1.2 -1.8 mW @ 20mA) for 10 minutes and the peak adsorption wavelengths were observed. Figure 2.16 displays the relationship between the peak adsorption wavelengths with solvent types.

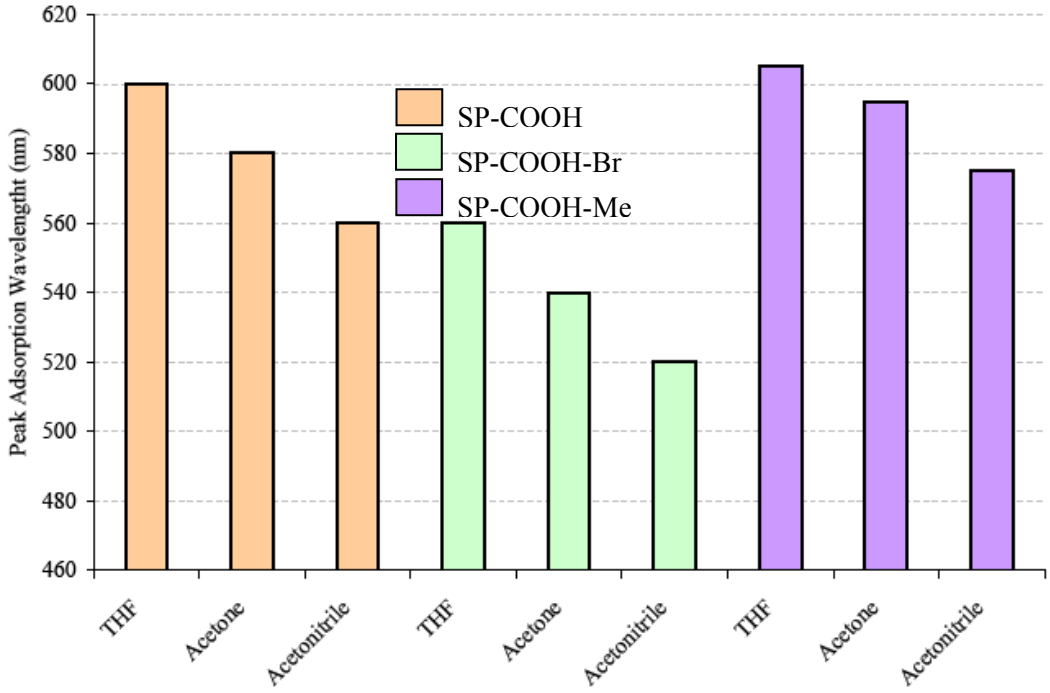


Figure 2.16 : Polar Aprotic Solvents Effect on Dye Photoswitching.

0.25 µg/ml of dye solutions at 20 °C were exposed to visible light and then UV light and the peak adsorption wavelength was noted (under UV illumination).

2.6.5 Aqueous Buffers (HEPES, PBS and MES) Effect on Dye Photoswitching

Based on the eventual application of the dyes (*i.e.* in conjunction with proteins in aqueous buffer solutions), the spiropyran ability to photoswitch in these solvents was observed. The dyes were attempted to be prepared in HEPES, PBS and MES (all at pH 7 and molar concentration of 0.1 M) but due to solubility limitation, the spiropyran dyes were eventually dissolved in ethanol and made up to the mass concentration of 0.25 µg/ml at a solvent ratio of ethanol: aqueous buffer (1: 9). The solutions were exposed to the white LED (27-33 cd @ 30mA) for 30 seconds and scanned between 400 and 700 nm using a UV-Vis Spectrophotometer. The solutions were then exposed to the UV LED (360nm 1.2 -1.8 mW @ 20mA) for 10 minutes and scanned. This was then re-introduced to white LED to established reversibility of switching, and scanned. However, the dyes failed to photoswitch upon exposure to the white LED and UV LED, they remained in a fixed state as evident in Table 2.5.

Table 2.5 : Dye Photoswitching via UV/Vis Illumination in Aqueous Buffers.

0.25 µg/ml (6.6 x 10^{-6} M) of dye solutions at 20 °C were exposed to visible light and UV light and then back in visible light. The absorption maxima were noted.

Spiropyran Dye	Solvent (pH 7) Ethanol : *Buffer* (1 : 9)	Visible Light Absorption maxima (nm)	UV Light Absorption maxima (nm)	Visible Light Absorption maxima (nm)
SP-COOH	HEPES	-	-	-
	PBS	-	-	-
	MES	-	-	-
SP-COOH-Br	HEPES	550	550	550
	PBS	550	550	550
	MES	550	550	550
SP-COOH-Me	HEPES	580	580	580
	PBS	580	580	580
	MES	580	580	580

As evident in Table 2.5, the synthesised dyes were locked in one isomer form or the other. SP-COOH remained in the spiropyran form regardless of illumination. SP-COOH-Br and SP-COOH-Me however remained in the coloured merocyanine form regardless of illumination. The UV LEDs light source was replaced by a higher intensity UV light source (Ultra Violet 350nm - Compact Fluorescent L Type 18 Watt 2g11 4 Pin Cap with suitable filter) in anticipation that the dye would photoswitch. It was however observed that the alternative UV irradiation was of no advantage. The dyes remained in the spiropyran form. Conversely, when the dark adaptation experiment was conducted on the same set of samples, SP-COOH showed indications of photoswitching. However the rate of change was slow as evident in Figures 2.17 and 2.18. SP-COOH-Br and SP-COOH-Me failed to switch isomer forms regardless of dark adaptation incubation period. The presence of water seems to have had an effect on the photoswitching properties of SP-COOH-Br and SP-COOH-Me. It was then concluded that if SP-COOH-Br and SP-COOH-Me can not be triggered by light stimulus, they would hence forth be excluded from the remainder of the study.

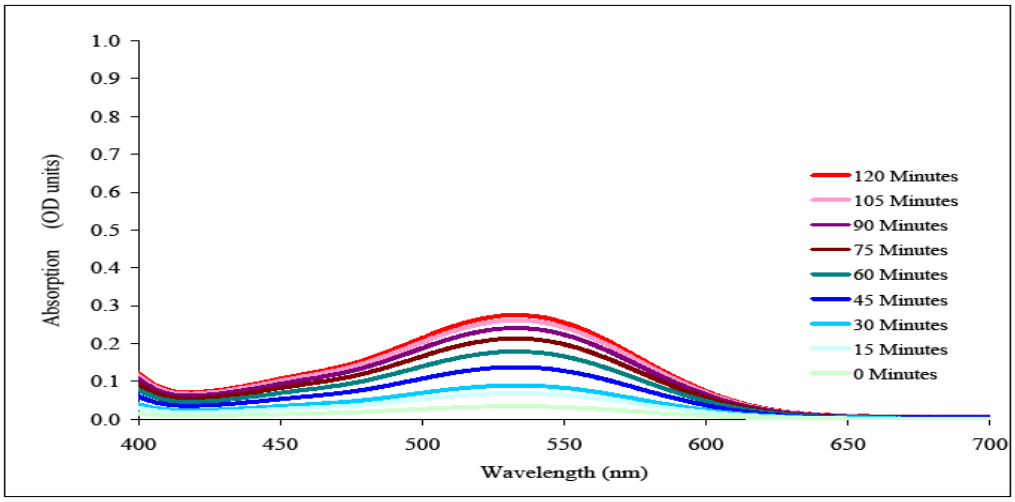


Figure 2.17 : Dark Adaptation Photoswitching of SP-COOH Over 2 Hours.

Photoswitching of the spiropyran form to the merocyanine form. Sample contained 0.25µg/ml (6.6 x 10^{-6} M) of dye in ethanol : HEPES (1: 9). Initial illumination with white LED for 30 seconds, then placed in the UV-Vis Spectrophotometer (dark environment), and scanned every 15 minutes for 2 hours at 20 °C.

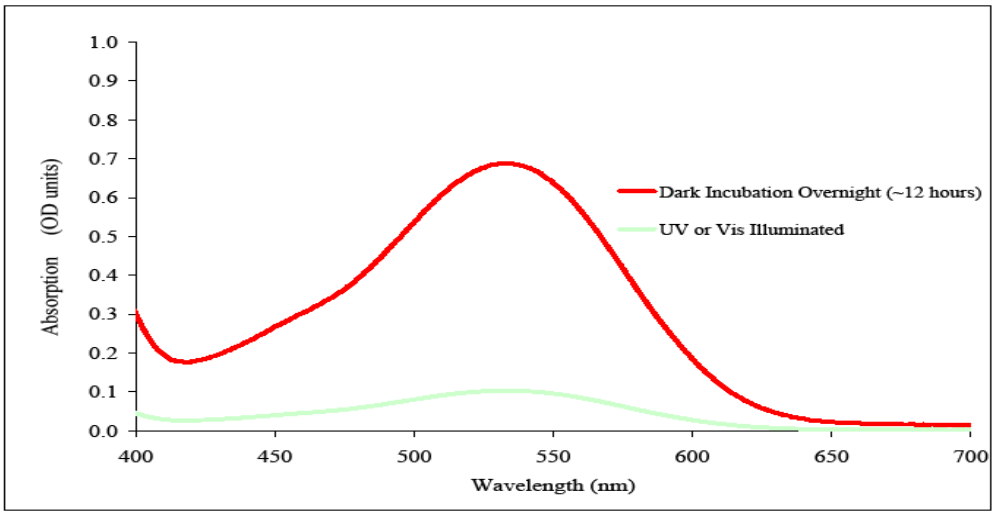


Figure 2.18 : Dark Adaptation Photoswitching of SP-COOH (~ 12 Hours)

Photoswitching of the spiropyran form to the merocyanine form. Sample contained 0.25µg/ml (6.6 x 10^{-6} M) of dye in ethanol : HEPES (1: 9). Initial illumination with white LED for 30 seconds, placed in the dark (at room temperature) overnight (~12 hours) and then scanned in the UV-Vis Spectrophotometer (without it being exposed to ambient light).

The PBS and MES buffers also displayed very similar results to the HEPES. Figures 2.19, 2.20, 2.21, 2.22 represent the dark adaptation experiment results at a ratio of ethanol to aqueous buffers maintained as 1:9.

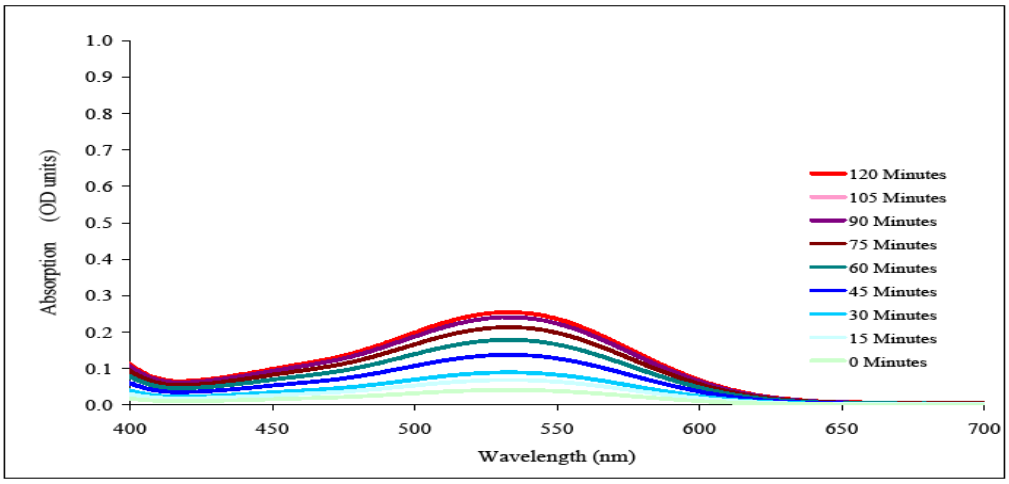


Figure 2.19 : Dark Adaptation Photoswitching of SP-COOH Over 2 Hours.

Photoswitching of the spiropyran form to the merocyanine form. Sample contained 0.25µg/ml (6.6 x 10^{-6} M) of dye in ethanol : PBS (1: 9). Initial illumination with white LED for 30 seconds, then placed in the UV-Vis Spectrophotometer (dark environment), and scanned every 15 minutes for 2 hours at 20 °C.

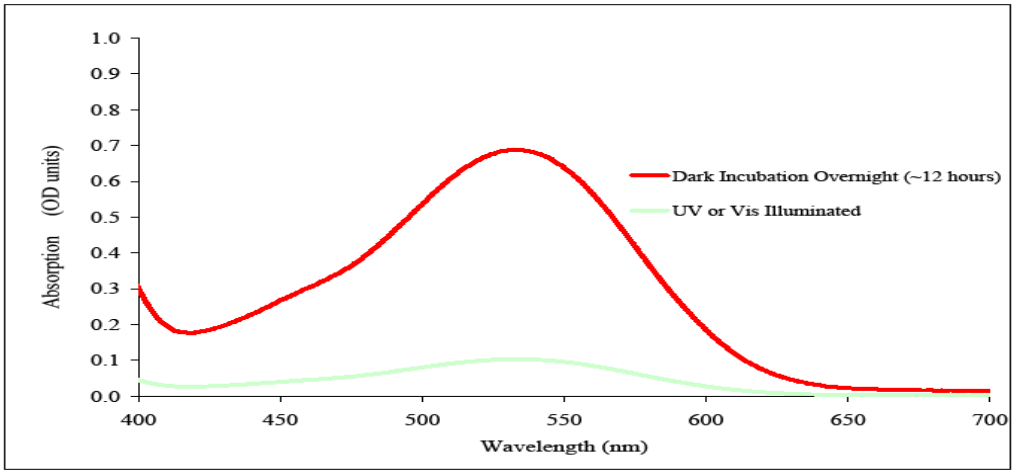


Figure 2.20 : Dark Adaptation Photoswitching of SP-COOH (~ 12 Hours)

Photoswitching of the spiropyran form to the merocyanine form. Sample contained 0.25µg/ml (6.6 x 10^{-6} M) of dye in ethanol : PBS (1: 9). Initial illumination with white LED for 30 seconds, placed in the dark (at room temperature) overnight (~12 hours) and observed in the UV-Vis Spectrophotometer (without it being exposed to ambient light).

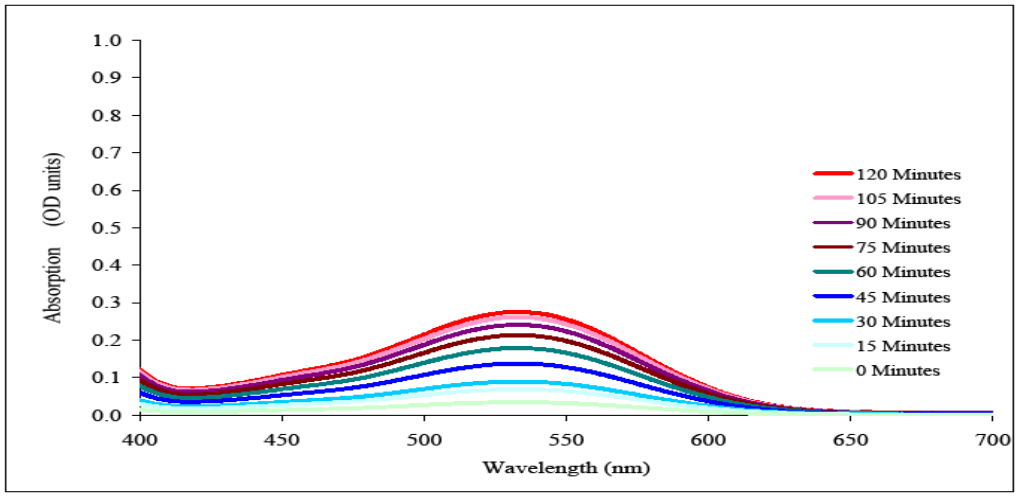


Figure 2.21 : Dark Adaptation Photoswitching of SP-COOH Over 2 Hours.

Photoswitching of the spiropyran form to the merocyanine form. Sample contained 0.25µg/ml (6.6 x 10^{-6} M) of dye in ethanol : MES (1: 9). Initial illumination with white LED for 30 seconds, then placed in the UV-Vis Spectrophotometer (dark environment), and scanned every 15 minutes for 2 hours at 20 °C.

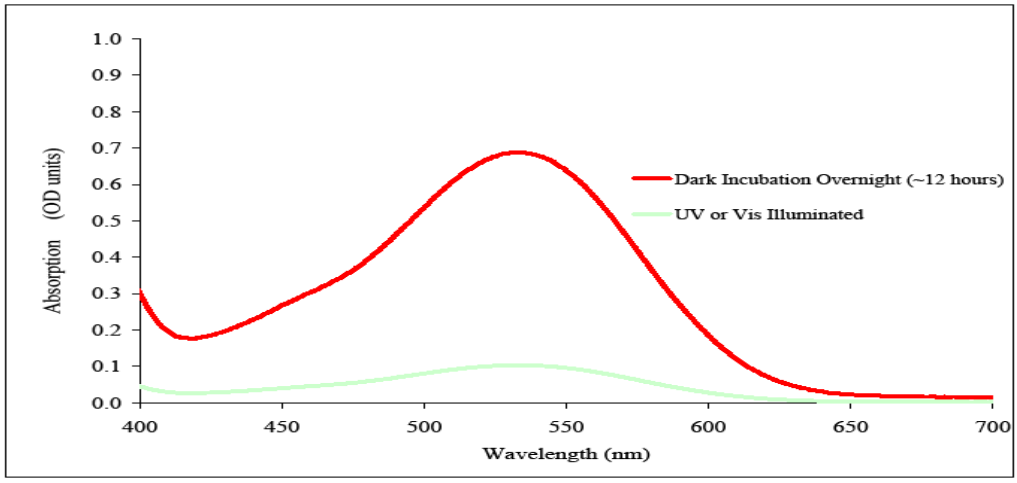


Figure 2.22 : Dark Adaptation Photoswitching of SP-COOH (~ 12 Hours)

Photoswitching of the spiropyran form to the merocyanine form. Sample contained 0.25µg/ml (6.6 x 10^{-6} M) of dye in ethanol : MES (1: 9). Initial illumination with white LED for 30 seconds, placed in the dark (at room temperature) overnight (~12 hours) and then scanned in the UV-Vis Spectrophotometer (without it being exposed to ambient light).

Having established the behaviour of the aqueous buffer on the synthesised dyes, an extended study of varying the ratio of ethanol to buffer 1:9, 1:5, 1:1, 5:1 and 9:1 was

performed to observe the change. HEPES was the chosen buffer of study (because of availability during study) observed in figure 2.23.

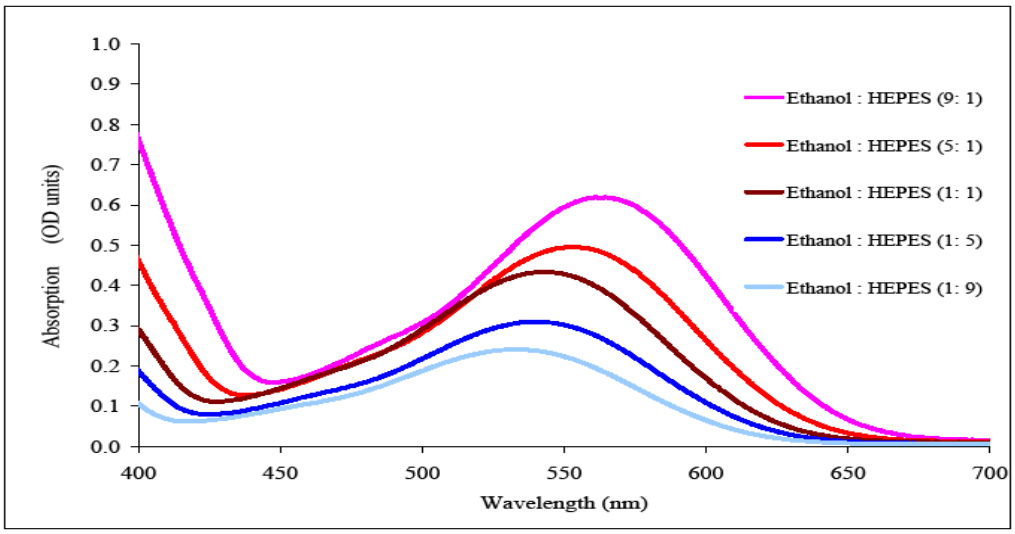


Figure 2.23 : Dark Adaptation Photoswitching at varying Ethanol: HEPES ratios.

Photoswitching of the spiropyran form to the merocyanine form. Sample contained 0.25µg/ml (6.6 x 10^{-6} M) of dye at various ethanol : HEPES ratios after 2 hours dark adaptation incubation. Ratios range through 1: 9, 1: 5, 1: 1, 5: 1 and 9: 1 of ethanol: HEPES respectively.

The peak adsorption shift as well as the optical wavelength in Figure 2.23 helps to establish the how solvent has a significant effect on the photowitching properties of the spiropyran dye.

Generally, the dark reaction is much slower which eliminates one of the properties desired; rapid switching. Nonetheless, the photoswitching capability of the SP-COOH dye was still functional although it seems that the dye absorption maxima underwent a hypsochromic shift from 565nm (ref. Figure 2.9) to 540nm most likely to have been caused by the interaction between the net polarity of the solvent components and the merocyanine. This effect is also visible in Figure 2.23.

From the observation within this study, in organic solvents such as ethanol, SP-COOH photochromically behaved as follows:

- Dark or UV illumination = Merocyanine form
- Visible light illumination = Spiropyran form

In aqueous buffer (ethanol: 0.1M, pH7, aqueous buffer) SP-COOH photochromincally behaved as follows:

- Dark = Merocyanine form
- Visible light or UV illumination = Spiropyran form.

2.7 Temperature and pH Effect on Synthesised SP-COOH

2.7.1 Introduction

Spiropyrans are known to also undergo thermochromism *i.e.* the thermally induced reversible colour change (Bouas-Laurent *et al*., 2001). Investigating this phenomenon not only helps confirm that the dyes are behaving as expected but more importantly having established the relationship between photochromism and solvent properties, (and having decided to involve the incorporation of ethanol: HEPES mix rather than just ethanol for the remainder of this study) it is appropriate to analyse to what extent or limitations the solvent effect have with regards to thermochromism. Furthermore the formation of merocyanine form of the dye is caused by the cleavage of covalent bonding as previously explained; hence, it was also ideal to know the extent of degradation (if any) by temperature on the synthesised spiropyran dye.

Likewise, pH variation is known to have an influence on a number of chemical reactions. Photochromism of spiropyrans are also suggested to be influenced by change in pH of the solvent (or the addition of an acid or base to the solvent). On pH change the dyes are thought to experience acidichromism and/or halochromism. Acidichromism phenomenon is defined when the protonated form and the conjugate base of the compounds have distinctly different absorption spectra (exampled by phenols and aromatic amines) (Bouas-Laurent *et al*., 2001). However with regards to photochromic dyes, this phenomenon can occur in addition to solvatochromism - hence the pH factor also help define conditions of use. Halochromism denotes the

colour change of a dye on addition of acids or bases. Its phenomenon is caused by the formation of new chromophores, as in acid-base indicators (Bouas-Laurent *et al*., 2001).

2.7.2 Temperature Effect on Synthesised Dye

Within this experiment, 0.25 µg/ml of dye was prepared in ethanol: HEPES (1: 9) by first dissolving the dye in appropriate amount in ethanol then 0.1M, pH 7, HEPES buffer was added. This was prepared in triplicate. Each solution was initially exposed to the white LED for 30 seconds to ensure spiropyran conversion. 1 ml was placed in a quartz cuvette (path length: 1 cm). The dye solution was observed at 540 nm using a UV-Vis Spectrophotometer. The solution was then placed in a water bath (at known temperature range: 20, 30, 40 50, 60, 70 and 80 °C) which was sealed off from ambient light and the solution was incubated 30 seconds. The solution was observed at 540 nm using a UV-Vis Spectrophotometer.

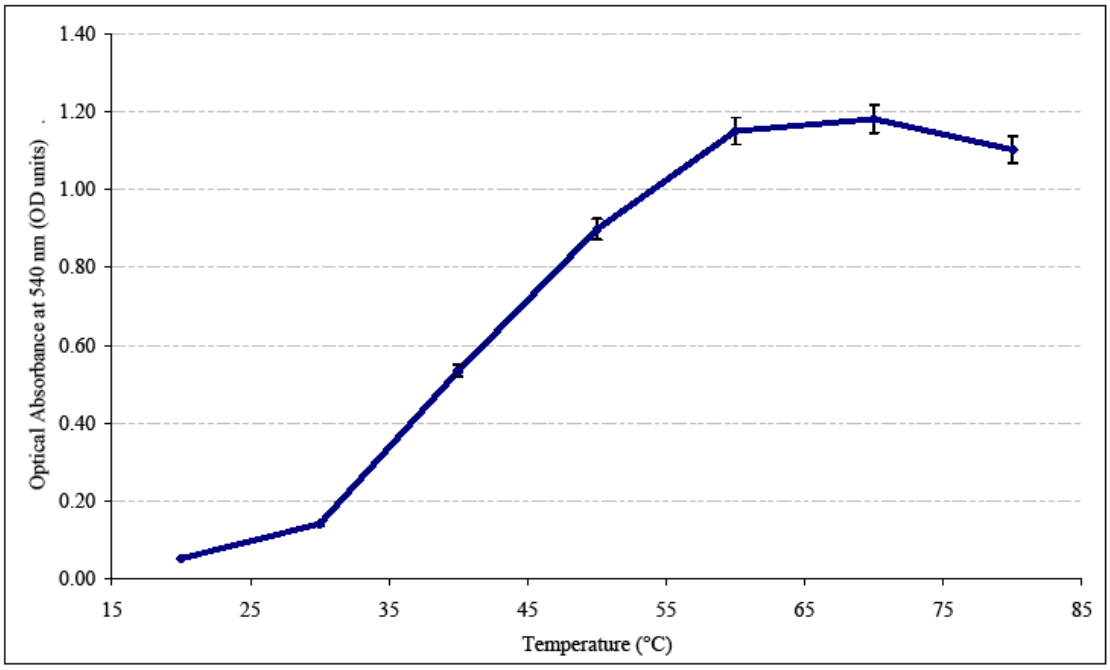


Figure 2.24 : SP-COOH Thermoswitching.

Thermoswitching of the SP-COOH at 20, 30, 40 50, 60, 70 and 80 °C. 0.25 µg/ml (6.6 x 10^{-6} M) of dye was in ethanol: HEPES (1: 9) observed OD at 540 nm.

Figure 2.24 is a plot of the OD observed at 540 nm at each specific pH and from the results attainted, it is evident (by the formation of the merocyanine form of dye), that thermochromism occurred with the synthesised dye and in the solvent ethanol: HEPES (1: 9). However, signs of degradation of the dye was also evident with further increase in temperature above 70 °C.

2.7.3 pH Effect on Dye Photoswitching

This study was set up to observe the nature pf pH effect on dye. The solvent pH was varied and the allowed to convert to the merocyanine form and OD observed. The OD represented the concentration of merocyanine present. 0.25 µg/ml of dye was prepared in ethanol: HEPES (1: 9) just as in the temperature related experiment. The solutions were then pH adjusted with HCl or NaOH to attain net solution pH values of 2, 3, 4, 5, 6, 7, 8, 9 appropriately (in triplicate). The solutions were initially exposed to the white LED for 30 seconds to ensure spiropyran conversion. 1 ml was placed in a quartz cuvette (path length: 1 cm). With appropriate solvents as blanks, the dye solution was the peak adsorption was observed at 540 nm using a UV-Vis Spectrophotometer. The samples were then placed in a dark environment at 20 °C overnight (~ 12 hours) to allow merocyanine conversion and observed at 540 nm using a UV-Vis Spectrophotometer.

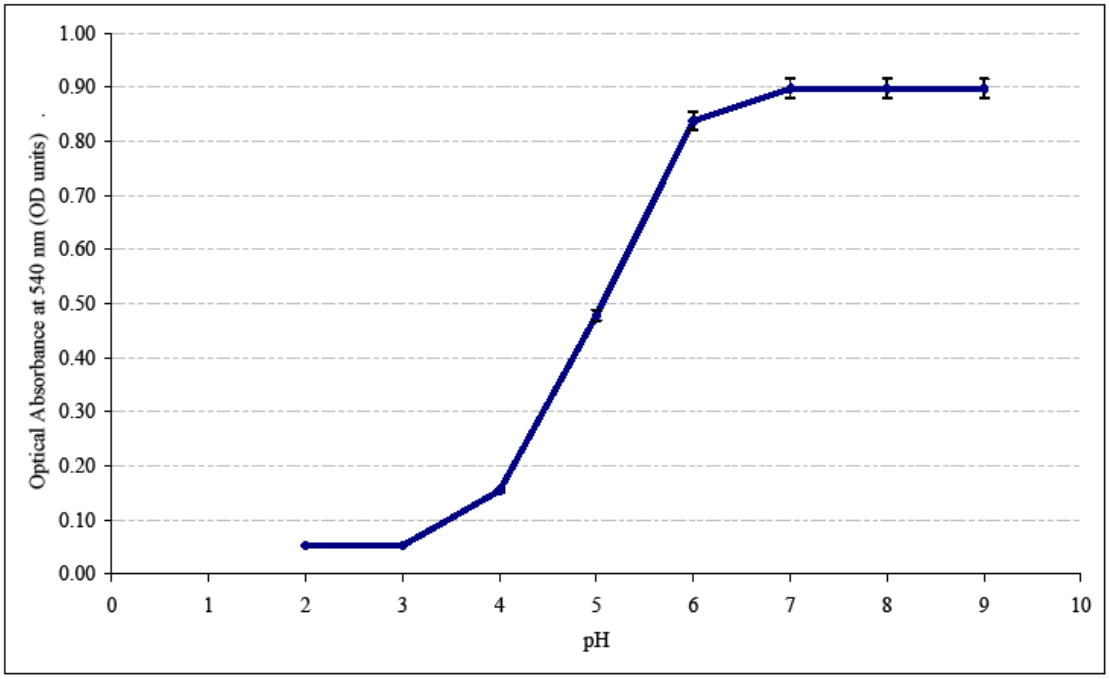


Figure 2.25 : pH Effect on Dye Photoswitching.

Photoswitching of the SP-COOH at pH values of 2, 3, 4, 5, 6, 7, 8, 9. 0.25 µg/ml (6.6 x 10^{-6} M) of dye was in ethanol: HEPES (1: 9) observed at 540 nm.

From Figure 2.25, it is observed that the dye at lower pH favoured the spiropyran form to the merocyanine form *i.e.* the dye remained in the spiropyran form of the dye regardless of illumintation. However increase in pH gradually restores the photochromic effect (*i.e.* the conversion into the two isomers state when illuminated appropriately). It was also noted that any further increase in pH (7 and above) did not have any further effect on the conversion of isomer state, *i.e.* when normal photochromism is attained at pH 7, further increase in pH did not have any (favourable or adverse) effect on photochromism.

2.8 Photodegradation (Fatigue) Assessment

2.8.1 Introduction

Photochromism (a non-destructive process), involves cleavage of covalent bonds and re-assembling of structure, however side reactions can occur during photoswitching

and as a result, loss of switching performance over time can lead to chemical degradation of the compound. This effect is also referred to as "fatigue". In comparison to material science, fatigue is the progressive and localised structural damage that occurs when a material is subjected to cyclic loading.

Despite the photoswitching stability of spiropyrans being improved by the introduction of a nitro group at the 6 position of the benzopyran moiety of the structure, spiropyrans are still subjected to photodegradation when in solution. The photo stability of the photochromic compounds has been widely studied in various literature but photochromic dyes are used under various conditions and states, hence it seems ideal to examine photodegradation of the synthesised dye under the intended environmental conditions (*i.e.* in solution of ethanol: HEPES ratio of 1: 9, pH 7). Photodegradation process is one of the major limiting factors for photochromic dyes in industrial applications (Baillet *et al.*, 1994).

2.8.2 Photodegradation (Fatigue) Assessment of SP-COOH

To assess whether the synthesised dye showed any degradation when constantly being changed form one isomer to another, SP-COOH was initially put through 10 cycles (of spiropyran / merocyanine conversion) spaced by 12 hour intervals (to allow dark adaptation to the merocyanine form). A second assessment which involved a longer stability assessment whereby SP-COOH was put through 10 cycles spaced by 1 week intervals. 0.25 μg/ml of dye was prepared in ethanol: HEPES (1: 9) just as in previous studies in triplicate. The samples were initially exposed to the white LED for 30 seconds to ensure spiropyran conversion. 1 ml was placed in a quartz cuvette (path length: 1 cm). The peak adsorption wavelength of the dye was observed at 540 nm. The samples were then places in a dark environment at 20 °C for 12 hours to allow merocyanine conversion and scanned again at 540 nm. This concluded the first cycle. The subsequent cycles were observed on the same batch of samples. Figures 2.26, 2.27 and 2.28 shows the fatigue relations with photoswitching cycles.

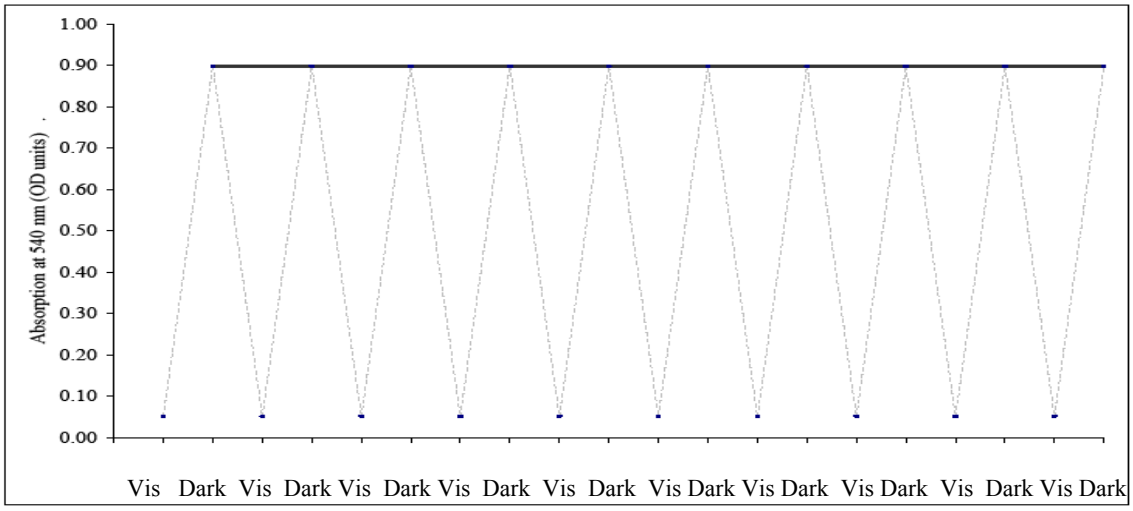


Figure 2.26 : Photodegradation of SP-COOH Over 10 Cycles (5 Days).

0.25µg/ml of dye in appropriate solution (ethanol: HEPES at 1: 9) was put through 10 cycles (of spiropyran / merocyanine conversion) spaced by 12 hour intervals (to allow dark adaptation to the merocyanine form) and scanned at 540 nm.

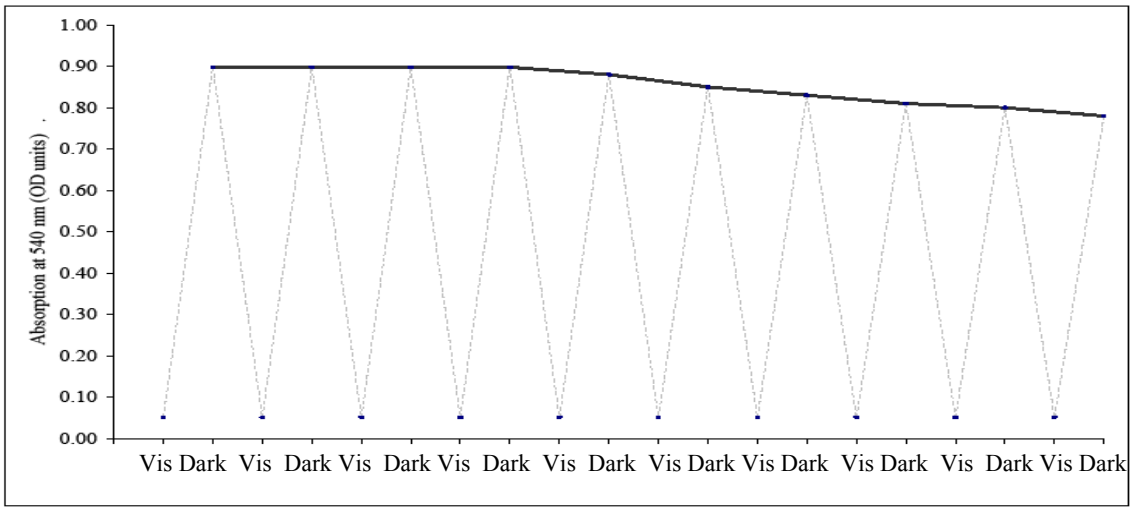


Figure 2.27 : Photodegradation of SP-COOH Over 10 Cycles (10 Weeks)

0.25µg/ml of dye in appropriate solution (ethanol: HEPES at 1: 9) was put through 10 cycles (of spiropyran / merocyanine conversion) spaced by 1 week intervals and scanned at 540 nm. This was a longer stability assessment.

Since the intended use with protein involves storage at 4 °C, the long time fatigue analysis was conducted at the lower temperate of 4 °C to certify if there would be any significant difference between storage at 20 °C and 4 °C.

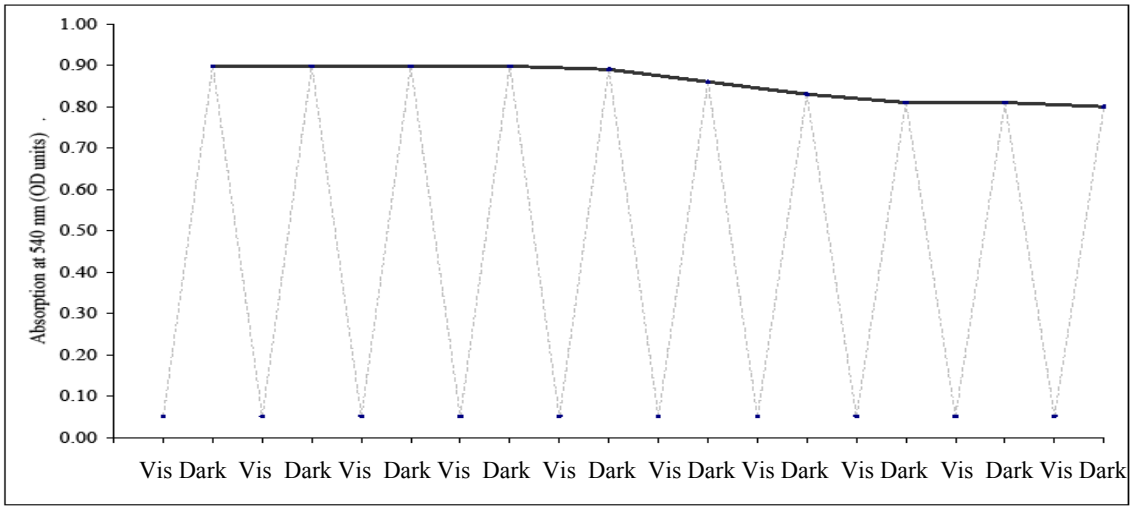


Figure 2.28 : Photodegradation of SP-COOH Over 10 Cycles (10 Weeks) @ 4 °C.

0.25µg/ml of dye in appropriate solution (ethanol: HEPES at 1: 9) was put through 10 cycles (of spiropyran / merocyanine conversion) spaced by 1 week intervals at 4 °C and scanned at 540 nm.

From figures 2.26, 2.27 and 2.28, it observed that the length of time between photoswitch cycles of the dye has no significant effect on fatigue. Although the test could have been prolonged to observe even longer term effect, it can be assumed that within this thesis, the synthesised dyes do not significantly undergo fatigue.

2.9 Discussion

2.9.1 Synthesis, Characterisation and Photoswitching of Carboxylated Spiropyran Dyes

The synthesis of the carboxylated spiropyran dyes were analysed by various means to check authenticity as well as purity.

With regards to characterisation via TLC analysis, the results indicated that the synthesised dyes were of relatively high purity, and as the initial confirmation of the identity of the synthesised dye, showed similarity (in R_f values) when compared to the commercially available spiropyran dye (SP-Sigma). 1D ^{1}H NMR study also confirmed the identity of the dye based on the number of hydrogen atoms present on a dye molecule. The hydrogen atom on the benzene rings of the spiropyran dye structure were accounted for by NMR, however not all were obvious. This then indicated that the purity of the dye was not as high as initially deduced from the TLC analysis. ^{13}C NMR could not however be completed on dyes. Initial trial analysis indicated the amount dyes needed to give significant detections was be at the expense of other experimental times and resources hence was not completed. It was however stated by Bouas-Laurent *et al.*, (2001) that ^{13}C NMR was not feasible within their analysis. IR spectroscopy was able to detect the C=O in the carboxyl functional group of the synthesised dye.

The set up of photochromic activity in ethanol involving the use of LEDs as illumination source was an avenue that was earnestly considered due to the advantages of LEDs consisted of compared to high power light sources such as mercury arc or tungsten lamps. The use of LEDs was to eliminate one major factor of heat energy been accompanied by visible light illumination, which was associated with factors that contribute to photodegradation. The initial results of photochromism of synthesised dyes in ethanol with LEDs as light source showed expected and successful photoswitching.

2.9.2 Solvent Effect on Synthesised Spiropyran Dyes

Environmental conditions such as the solvent compositions and polarity can have an effect on the photochromic activity of the spiropyran dyes. Generally spiropyran are expected to display normal photochromism (the most thermodynamically stable isomer of the dye being the spiropyran form) in non polar organic solvents. In polar organic solvents, depending on the polarity of the solvents, the dyes may display negative photochromism (the most thermodynamically stable isomer of the dye being the merocyanine form). However the carboxylated dye is known to display negative photochromism in solution because of the presence of the COOH functional group, and this was evident in the dark adaptation reaction – *i.e.* the dye was thermodymically stable in the merocyanine form in the dark.

The polarity of the solvent affected the photoswitching profile of the synthesised dye. As stated by Lin (2003) - with increasing solvent polarity, the absorption band would be expected to undergo a hypsochromic shift because there would be extra interaction between polar solvents and ionic coloured merocyanine form. This was also evident with the results attained, i.e. as the dielectric constant increased within grouped organic solvent type, the hypsochromic shift was observed regardless. As theoretically established in Chapter 1, merocyanines may be cis (C) and/or trans (T) forms of the isomer. The cis form being unstable and hence will either transform into the trans structure or revert to spiropyran depending on the light and solvent conditions. However the results attained in this study did not necessarily agree with Lin, (2003) theoretical explanations neither did it give more insight on which form of the merocyanine form is present in each solvent. Lin, (2003) concluded that in a polar solvent, the maximum absorbance occurs at lower energy, causing a Bathochromic (red) shift. As a results of these hypothesis, in a non-polar solvent as well as aprotic solvent, (in reference to the proposed isomer configuration in the merocyanine – section 1.3) TTC and TTT are acticipated to be dominant, whereas in protic solvent, CTT and CTC are acticipated to be dominant.

Aqueous buffer effect of the dye however gave results that influenced the approach of the dye application. HEPES, PBS and MES buffers of choice observed. HEPES and MES can be applied in EDC-NHS reactions and PBS is the base medium in which most antibodies are presented although not an exclusive solvent since its isotonic properties is its main advantage. Nevertheless, the synthesised dyes failed to easily dissolve in these solvents and as results the dyes had to been initially dissolved in ethanol then further made up with the aqueous buffer. The use of UV LEDs and higher intensity UV light source were noted as innaporpriate when the aqueous buffer were involved as solvent types.

The desired characteristic of photochromic dyes; possession of zero or minimal internal filtration mechanism to inhibit efficacy of activation photon was not met with the introduction of aqueous solvents as mediums. The only option of dark adaptation switching (reliance on the negative photochromic nature to switch to merocyanine form in the dark) was only applicable to SP-COOH dye. The remaining derivatives SP-COOH-Br and SP-COOH-Me were discarded for the remainder of the study since they failed to change form regards of illumination or dark adaptation incubation period. Varying the ratio of ethanol to HEPES resulted in a hypsochromic shift. In comparison to Gorner *et al.,* (1998) observation, they also noted that the addition of water to the spiropyran dye (in acetone) resulted in blue shift of from 580 to 540 nm. Collectively, this shows that the photo induced structural changes depend on solvent composition; hence the photo response can be modulated by a combined action of light and solvent composition. HEPES, MES and PBS all behaved identically.

2.9.3 Temperature and pH Effect on Synthesised Spiropyran Dye

Temperature effect (thermochromism) on the synthesised dye was as expected. As temperature increases more merocyanine became present in the solution denoted by the deep colouration with time. The solvent choice *i.e.* the use of ethanol: Hepes (1: 9) did not have any effect on thermochromism per se as did in photochromism. However the dye degraded rapidly with thermochromism. This is also a problem when it comes to proteins (the intended application), heating at such high temperature will cause denaturing. The stability of the dye is highly influenced by thermochromism. But in

the general context of thermochromism, SP-COOH in desired solvent composition (ethanol: Hepes (1: 9)) displayed the effect as expected.

Gallot *et al.*, (1997) stated that when spiropyrans are treated with acids they are converted into spiropyran salts which exhibit the photochromic behaviour. Keum *et al.*, (1994) also reported that treatment of the spiropyran form in an organic solvent with HCL to decrease the pH causes a protonated spiropyran form of the dye to ring open to give a protonated merocyanine form. Treatment of the latter with a tri-n-butylamine solution as base in a stopped-flow apparatus resulted in ring closure to spiropyran reconversion. This was however not so in this current study. The pH change exposed the near opposite and slightly different effect possibly based on the solvent involved (ethanol: HEPES at 1:9). At lower pH, the dye is immediately converted to the spiropyran form and does not respond to photo or thermo chromism. The dye remains locked in that form regardless of the external stimuli. With the addition of a base to increase the pH of the solution, the photochromic nature is restored i.e. the system responds to change in illumination (Photochromism). Hence once again collectively the photo induced structural changes depend on solvent composition and its environmental conditions such as pH and temperature. Hence these results help to define the synthesised dye photochromic activity based on its intended application.

2.9.4 Photodegradation (Fatigue) Assessment of Spiropyran Dyes

Photodegradation of the synthesised dye in ethanol: HEPES (1: 9) at pH 7 was observed under three conditions. The first being immediate switching the dye isomeric states over ten cycles at 20 °C bearing in mind that UV illumination to aid switching was no longer feasible. The dye in such solvent composition rendered slow dark conversion rate from the spiropyran form to the merocyanine form which was possible within 12 hours. The second observed condition was in the context of stability of the dye over time. The ten cycles switching of the dye over 10 weeks (~ 3 months) period showed that after the fifth week, the dye began to show some form of degradation although not very drastic. The third observed condition took on board the possible storage condition of the dye in solution (at 4 °C) when it is applied on

proteins, and the stability of the dye over time was observed. The results however did not differ from the stability test over time at 20 °C.

These results however confirm the synthesised dye in ethanol: HEPES (1: 9) at pH 7 did not show degradation within 10 completed cycles or after 5 weeks of storage. They also contrast reported results on the stability of photochromic property in spiropyran dye as relatively poor. However it must be noted that habitually these results stated are with respect to dye–protein complexes which will be later looked into in subsequent chapters.

2.10 Summary of Dye Synthesis, Dye Characterisation, Dye Photoswitching Conditions and Fatigue

- Spiropyran dye synthesis to possess a carboxyl group has been successfully completed.
- The synthesised dye illustrated photochromism and was structurally confirmed with TLC, NMR and IR analysis.
- Photochromic behaviour of the dye in various polar and non polar solvents confirmed variance in photochromism with respect to environmental conditions. In aqueous solvents (proposed application environment) the photochromic nature of the dye was affected and as a result the dye had to forgo its rapid switching photochromic property, also photoswitching was triggered by visible light and the reverse reaction by dark adaptation.
- The synthesised spiropyran dye was relatively stable (when stored in solution for more than a month) and did not show fatigue with within 10 cycles of photoswitching.

Chapter 3

PHOTOMODULATION OF HORSERADISH PEROXIDASE ACTIVITY MODIFIED WITH SPIROPYRAN DYES

3 Photomodulation of Horseradish Peroxidase Activity Modified with Spiropyran Dyes

3.1 Introduction and Overview

This chapter first of all describes the conjugation of horseradish peroxidase (HRP) to the synthesised carboxylated spiropyran dyes (SP-COOH) (of Chapter 2). This is then followed by characterisation of the spiropyran modified HRP (SP-HRP). Within this chapter, the results of the conjugation reaction between HRP and SP-COOH will be presented in the characterisation sections and not the conjugation section. This is because coupling reactions were conducted in a way to assist the characterisation of the modified enzyme.

The concept of photomodulating enzyme activity has been successfully studied by a small number of research groups (examples presented in Chapter 1, Section 1.2.4.2 and Table 1.1), of these, Weston *et al*., (1999) reported the greatest degree of photomodulation of HRP (greater than 90% reduction in enzyme activity under visible compared to UV illumination). The hypothesis put forward by Weston *et al*., (1999) states: spiropyran dyes attached to the enzyme HRP alters physicochemical properties of the entire molecule upon appropriate irradiation; which has the potential to disrupt the tertiary structure of the protein, thus possibly inducing a subsequent change in the configuration of the binding or active site of the protein.

As established as part of the aims and objectives within this thesis, HRP is studied here as a model system to develop and demonstrate the approach to be later applied during photomodulation of antibodies. This includes observing a conjugation reaction method accredited by the number of spiropyran dyes effectively attached to the enzyme; observing photomodulation of the enzyme activity under experimental settings favourable to antibodies (*i.e.* in an aqueous environment); and observing photoswitching capabilities (including fatigue) of the spiropyran dye on the modified enzyme. HRP was chosen as it is a well studied and readily available enzyme. Its activity can be simply assayed using colorimetric reagents. In addition, HRP has a

molecular size similar to an antibody fragment (~ 44 k Da); a candidate included in antibody study.

Within this current study (based on the findings related to photoswitching feasibility of spiropyrans in predominantly aqueous solvents [Chapter 2]), it was established and concluded that UV illumination can no longer be an effective stimulus for switching spiropyrans dyes to the merocyanine form, the dark adaption mechanism was henceforth applied. Likewise, the choice of aqueous buffer used through out experimentation was HEPES (simply because of availability; having also established and concluded that HEPES, PBS and MES buffers did not have any significant differing effect on the photoswitching properties on the spiropyran dye [Chapter 2]). The most applied conjugation method of coupling photochromic molecules to proteins: carbodiimide mediated coupling chemistry was used as with other research groups (Chapter 1; Table 1.1).

3.2 Chemicals, Biological Compounds, Materials and Equipment

The following chemical consumables were purchased from Sigma Aldrich, UK: Peroxidase from Horseradish (Type II) (Sigma Code: P-8250), N-Hydroxysulfosuccinimide Sodium Salt (Sigma Code: 56485), 1-(3-Dimethylaminopropyl)-3-Ethylcarbodiimide, Polymer-Bound (Sigma Code: 424331), Phosphate Buffered Saline (Sigma Code: P4417), HEPES Buffer Salts (Sigma Code: H7523), 3,3',5,5'–Tetramethylbenzidine (TMB) Tablets (Sigma Code: T 5525). Pierce Fluoraldehyde™ Reagent Solution (Pierce code: 26025) was purchased from Pierce, UK. The following were obtained from Fischer Scientific, UK: Centrifugal Concentrator Millipore Microcon YM-10 green 10,000 NMWL 0.5mL (Fischer Code: Fdr-561-030u) and Syringe Filter Non Sterile SFCA membrane green 25mm diameter 0.45µm pore size NALGENE (Fischer Code: Fdm-345-020k). LEDs were acquired from Roithner Laser Technik, Austria; White LEDs, based on GaN 27-33 Cd (Roithner Laser Technik Code: 5w4hca-H20-Ultra). UV-Vis spectra and protein assays were recorded on UV-Vis Spectrophotometer UV-2100 (Shimadzu), and Cary Eclipse Fluorescence Spectrophotometer (Varian) respectively.

3.3 Covalent Coupling of SP-COOH to HRP

3.3.1 Introduction

To test the feasibilty of HRP activity photomodulation, the carboxyl group of the synthesised spiropyran dye is initially coupled to the accessible lysine amine groups of the HRP molecule. This is achieved via covalent coupling. Water-soluble carbodiimides are often used as zero-length cross-linkers in organic chemistry or peptide synthesis for; attaching haptens to carrier proteins to form immunogens, to label nucleic acids and for the immobilization of different biological molecules on solid supports, amongst other applications Wrobel *et al*., (2002). In principle the carbodiimide molecule reacts with the carboxyl group of a molecule, producing a highly reactive *O*-acylisourea intermediate, which then further reacts with nucleophiles such as primary amines to form an amide (peptide) bond. The reaction can be performed in an aqueous solution at room temperature, which simplifies the procedure. Figure 3.1 shows a schematic representation of the carbodiimide mediated reaction.

Over the years, 1-(3-Dimethylaminopropyl)-3-Ethylcarbodiimide (EDC) has emerged as the carbodiimide reagent of choice for amide coupling reactions. EDC has a very short hydrolysis half-life, it is also highly reactive but unstable. If its reactive intermediate *O*-acylisourea (during an amide coupling reaction) does not encounter an amine, it hydrolyzes and regenerates the carboxyl group. However in the presence of *N*-hydroxysulfosuccinimide (Sulfo-NHS), carboxyl groups react with Sulfo-NHS (in the presence of EDC) resulting in a semi-stable Sulfo-NHS ester, which may then react with primary amines to form amide cross-links (Figure 3.1). Sulfo-NHS presence stabilises the intermediate product and greatly enhances coupling efficiency Wrobel *et al*., (2002). EDC and Sulfo-NHS are soluble in aqueous and organic solvents. However, the incorporation of Sulfo-NHS also preserves and increases water-solubility of the modified carboxylated molecule. The inclusion of Sulfo-NHS makes the coupling reaction a two-step process; the first being an activation step, and the second being the joining step. Sulfo-NHS esters are sufficiently stable but hydrolyse within hours or minutes, depending on the water-content and pH of the

reaction solution (NHS esters have a half-life of 4-5 hours at pH 7, 1 hour at pH 8, and only 10 minutes at pH 8.6.). Hence, within this study, the reaction during the first step contained a solvent medium (HEPES) at pH 4. The pH was then raised to 7 during the second step and the NHS-activated molecules were quickly reacted with the amine containing target.

Furthermore, during an EDC/Sulfo-NHS coupling reaction, a by-product isourea is required to be separated from the reactant and product mix. This usually involves extraction of the by-product into an acidic aqueous phase, which may or may not have an effect on the product. This aqueous work-up can be avoided by employing EDC attached to a large polymer. The commercially available polymer bound EDC (P-EDC) (Figure 3.2), behaves similarly to EDC in solution, but the by-products of the reaction would remain on the polymer. The product, therefore, can be isolated simply by filtration and evaporation of the filtrate. Hence one of the benefits of P-EDC is that work-up of the reaction can be reduced to simple techniques such as filtration and evaporation, providing a cleaner, faster and easier method of coupling Desai *et al*., (1993). P-EDC was used during the coupling reaction in this study.

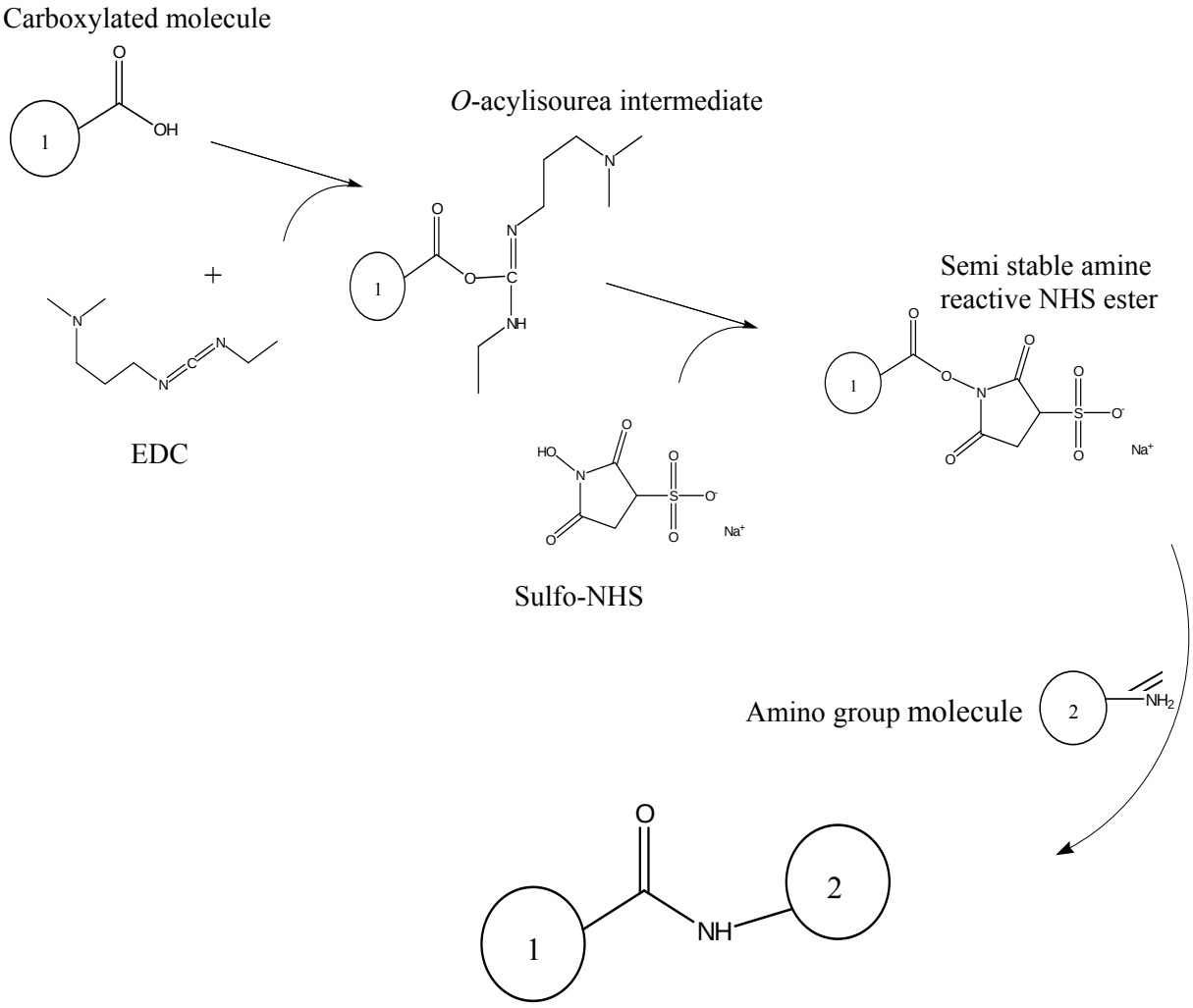


Figure 3.1 : Schematic of EDC/Sulfo-NHS Reaction.

EDC reacts with a carboxyl group on molecule #1, forming an amine-reactive *O*-acylisourea intermediate. This intermediate may directly react with an amine on molecule #2 (not shown in diagram), yielding a conjugate of the two molecules joined by a stable amide bond. However the inclusion of Sulfo-NHS (as conducted in this study) stabilises the amine-reactive intermediate by converting it to an amine-reactive Sulfo-NHS ester, thus increasing the efficiency of EDC-mediated coupling reactions.

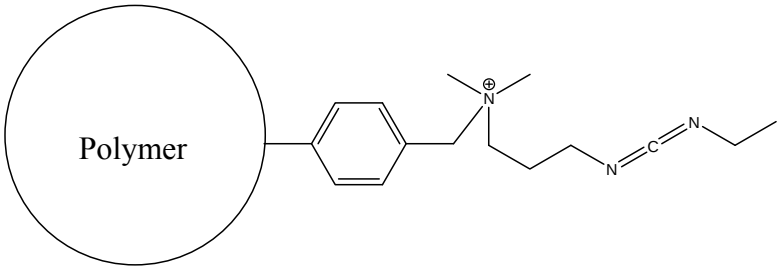


Figure 3.2 : Structure of Polymer Bound EDC.

Several agents can be classified as polymeric beads which are attached to EDC to form the polymer bound EDC. Examples are polymer linked nitrophenol derivatives, polystyrene-attached 1-hydroxy-2-pyrrolidinone, polymer coupled 8-acyloxyquinolin, polymer carrying N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline functional group, polymer attached to triphenylphosphine, etc. In this study the polymeric beads attached to EDC is chloromethylated poly(styrene-1% divinylbenzene) resin.

3.3.2 Methodology: Covalent Coupling of SP-COOH to HRP

Within this thesis, water based coupling of spiropyran dyes to protein molecules was achieved using the EDC/Sulfo-NHS reaction described in Section 3.3.1. This general procedure was followed during all spiropyran dye to enzyme coupling. Detailed protocol is as follow:

- EDC and Sulfo-NHS were initially equilibrated to room temperature.
- 1 mg of carboxylated spiropyran dye (SP-COOH) was weighed and initially dissolved in 1 ml ethanol. The solution was then made up to a volume of 10 ml with freshly prepared 0.1M Hepes buffer in ethanol (ethanol:HEPES; 1:9, at pH 4).
- 20 mg of P-EDC (0.5 mol/g of load contained EDC) and 5 mg of Sulfo-NHS (both these in molar excess quantities) were added to the dye solution (under ambient light conditions) and allowed to react for 15 minutes at room temperature (~ 20 °C).
- The solution was then filtered with a non sterile syringe filter (SFCA membrane, 25 mm diameter 0.45 μm pore size) to remove all by-products from the mixture.

- The pH of the solution was raised to 7 with NaOH and 1 mg of HRP was added and reacted for 2 hours (the length of time was varied in a rate of conjugation test later described).
- To quench the reaction, the solution was passed through a centrifugal concentrator (Millipore microcon 10,000 NMWL 0.5mL) with a molecular weight cut-off of 10 kDa and spun to dryness at 10,000 rpm for 15 minutes.
- The membrane surface of the centrifugal concentrator (which retained the modified HRP molecules) was reconstituted with a solution of 1:9 (ethanol:HEPES at pH 7) to collect the spiropyran dye labelled HRP (SP-HRP).

3.4 Characterisation of SP-HRP

3.4.1 Overview

To characterise the modified enzyme (SP-HRP), the following were observed: determination of the number of spiropyran dyes immobilised onto the enzyme expressed in molar ratios; photomodulation of the modified enzyme's activity; the correlation between photoswitching properties of the attached spiropyran dye (to the enzyme) and photomodulation; and spiropyran dye fatigue assesement.

3.4.2 Number of Spiropyran Dyes Attached to HRP (SP-COOH:HRP Ratio)

3.4.2.1 Introduction

In an attempt to understand the nature of coupling with regards to the number of spiropyran dyes attached to HRP, two investigations were carried out. The first study was to determine whether the reaction time during EDC/Sulfo-NHS coupling reaction had any significant effect on the amount of dye covalently attached to the protein. The second was to investigate whether the structural form of the dye (*i.e.* the spiropyran or merocyanine form) had any significant effect to the nature of coupling, because the two forms merocyanine and spiropyran are known to have two distinct structures and vary in properties (described in Chapter 1).

Characterisation by the number of spiropyran dyes immobilised onto the enzyme involved protein content assay and spiropyran dye concentration determination. Pierce Fluoraldehyde™ protein/peptide assay is employed in the protein content study. This method uses an o-phthalaldehyde-based reagent developed to detect minute amounts of protein and peptides. Fluoraldehyde reactions are completed in less than one minute with sensitivity down to 50 ng/ml protein concentration. The procedure also requires as little as 50 µl of the sample for use in an opaque microtitre assay plate; saving valuable sample and analysis time. Fluoraldehyde assay requires an excitation wavelength of 340 nm and emission wavelength of ~ 455 nm. It has been suggested that merocyanine form of the spiropyran dye is subjected to fluorescence emission at 610 nm whereas spiropyrans are not (Mecheri *et al.*, 2003). Pierce Fluoraldehyde™

protein/peptide assay employed does not involve fluorescence emission measurements at 610 nm. However, in order to rule out any ambiguity in the protein assay results within this study, the solution samples were all analysed in the spiropyran form; *i.e.* the samples were exposed to white LEDs for 3 minutes prior to analysis. Spiropyran dye concentrations were observed in the coloured merocyanine state because it shows strong absorption of radiation in the visible light spectrum region. The absorption maxima of the spectrum were used to provide the dye's molar extinction coefficient via a standard curve. The molar extinction coefficient was used to calculate the dye's concentration.

3.4.2.2 Methodology: Quantification SP-COOH & HRP

The spiropyran dye concentration of the modified protein was quantified as follows:

- SP-HRP sample was ensured to be in the merocyanine state (therefore pre-stored in a dark environment at 4 °C for 12 hours) (as described in Chapter 2, Section 2.5).
- The sample (SP-HRP) was observed under visible light absorption spectrum between 400 and 600 nm using a UV-Vis Spectrophotometer. (The absorption maximum was noted).
- With a calibration (standard) curve analysed beforehand, the molar extinction coefficient (ε) was calculated for the dye in solution and used to determine the spiropyran dye concentration of SP-HRP. In this study $\varepsilon = 4 \times 10^4\ M^{-1}cm^{-1}$ (at 510 nm in ethanol:HEPES [1:9])

Protein assay (Pierce Fluoraldehyde™ protein/peptide assay) was conducted as follows:

- SP-HRP sample was exposed to white LEDs light (as described in Chapter 2, Section 2.5) for 3 minutes to ensure spiropyran form conversion.
- 50µl of SP-HRP sample plus 50µl of Pierce Fluoraldehyde™ reagent (1:1 ratio) were pipetted in an opaque microtitre plate.

- The contents were mixed for 5 minutes and read on the Cary Eclipse Fluorescence Spectrophotometer after being excited at 340 nm, the emission spectra reading was between 450 and 460 nm. The results were compared to a calibration (standard) curve also previously analysed.

3.4.2.3 Experimental: EDC/Sulfo-NHS Rate of Coupling

Covalent coupling of SP-COOH to HRP was carried out as described (in Section 3.3.2); however this analysis involved the observation of the number of spiropyran dye attached to enzyme in different EDC/Sulfo-NHS reaction time. All reactions were in triplicates and averaged. The investigation was intended to observe whether reaction time had any significant effect on number of dyes coupled to the enzyme. The results of these were espressed as reaction time versus the molar ratio of spiropyran dye to enzyme in the conjugated SP-HRP (Table 3.1).

Table 3.1 : Rate of Formation of SP-HRP in an EDC/Sulfo-NHS Reaction.

Effect of reaction time on the carbodiimide-mediated covalent coupling of SP-COOH to HRP; expressed as reaction time versus the molar ratio of spiropyran dye to enzyme in the conjugate SP-HRP. Reaction time monitoring excluded the centrifugation step of 15 minutes during the reaction termination step (as described in Section 3.3.2).

Reaction time (minutes)	HRP:SP-COOH (molar ratio)
5	1: 6.3 (± 0.3)
20	1: 8.2 (± 0.2)
40	1: 9.1 (± 0.4)
60	1: 10.1 (± 0.2)
90	1: 10.3 (± 0.1)
120	1: 10.3 (± 0.1)

Table 3.1 shows that as the reaction time increased the number of moles of spiropyran dye per enzyme increased. It was however noted that after a 60 minutes reaction

period, the number of moles of dyes immobilised on the HRP enzyme did not significantly increase much further. Based on the fact that a HRP molecule possesses 12 lysine amine groups (as stated in Chapter 1; Section 1.5.4), a molar ratio of 1:12 would imply 100% SP-COOH immobilisation on the enzyme. However an initial deduction from thes results implies that, despite HRP possessing 12 lysine amine groups, not all may be accessible, since the spiropyran dye and the carbodiimide-mediated reagent involved in the reactions were all in molar excess to ensure reaction completion.

Another batch of conjugation under the same conditions but without EDC/Sulfo-NHS and in triplicates were conducted to investigate evidence of non-specific binding between HRP and SP-COOH. The results were also expressed as a function of time reaction.

Table 3.2 : Non-specific Binding Analysis Between HRP and SP-COOH.

Non-specific binding was assessed via mixing HRP with SP-COOH under the same conditions as the carbodiimide mediated reaction but without EDC/Sulfo-NHS. Results expressed as the molar ratio of enzyme to immobilised dye after coupling process. Reaction time stated below excludes the centrifugation step of 15 minutes to terminate the reaction.

Reaction time (minutes)	HRP:SP-COOH (molar ratio)
5	1: 2.2 (± 0.1)
20	1: 2.5 (± 0.1)
40	1: 2.6 (± 0.1)
60	1: 2.4 (± 0.1)
90	1: 2.4 (± 0.1)
120	1: 2.7 (± 0.1)

Results from Table 3.2 indicated that there may be some evidence of the presence of SP-COOH after the reaction, suggestion non-specific binding. It was however not verified via appropriate analysis whether the dyes were physically attached to the

enzyme or just present in the sample solution as a result of experimental error. Nevertheless, for an accurate representation of a *covalently* coupled reaction, the results attained in Table 3.1 were adjusted by subtracting the results from Table 3.2 to give Table 3.3.

Table 3.3 : Adjusted results of Table 3.1.

The molar ratio values of Table 3.1 minus the molar ratio values of Table 3.2, were used to construct this table.

Reaction time (minutes)	HRP:SP-COOH (molar ratio)
5	1: 4.1 (± 0.3)
20	1: 6.3 (± 0.2)
40	1: 7.5 (± 0.4)
60	1: 7.7 (± 0.2)
90	1: 7.9 (± 0.1)
120	1: 8.0 (± 0.1)

It was conluded that, the despite these adjustments, the initial results in Table 3.1 would be referenced through out this chapter, however the results in Table 3.3 will be presented in this thesis should future work clarify these observation.

3.4.2.4 Experimentation: Merocyanine and Spiropyran Effect on Coupling Reaction

Covalent coupling of SP-COOH to HRP was also observed by the state of the dye during coupling, *i.e*. the merocyanine or the spiropyran form, marked by dark reaction and visible light reactions respectively. The merocyanine dye was pre-incubated in the dark for 12 hours before application of the EDC/Sulfo-NHS reaction, whilst the spiropyran form of the dye was illuminated with white LEDs for 3 minutes to ensure isomer conversions. The coupling reactions were carried out in the dark and another in the presence of visible light from white LEDs. Since it has been established and confirmed in Section 3.4.2.3 that after 60 minutes of reaction the number of dye

attached to the enzyme did not significantly increase, the analysis was conducted till the 60 minutes mark. All reactions were in triplicates and averaged. Table 3.4 shows the reaction observed as dye isomer state versus reaction time versus the molar ratio of spiropyran dye to enzyme in the conjugated SP-HRP.

Table 3.4 : Merocyanine and Spiropyran Effect on Coupling Reaction.

Effect of dye's isomer state on the carbodiimide-mediated covalent coupling of SP-COOH to HRP expressed as the result of molar ratio of enzyme to immobilised dye. Reaction time excluded the centrifugation step of 15 minutes during reaction termination.

Dye's Isomer State	Reaction time (minutes)	HRP:SP-COOH (molar ratio)
Spiropyran	5	1: 6.1 (± 0.1)
	20	1: 8.0 (± 0.3)
	40	1: 8.9 (± 0.1)
	60	1: 10.2 (± 0.2)
Merocyanine	5	1: 6.0 (± 0.2)
	20	1: 8.7 (± 0.1)
	40	1: 9.4 (± 0.2)
	60	1: 9.8 (± 0.1)

The result shown in Table 3.4 implies that the nature of the dye isomer (*i.e.* either spiropyran or merocyanine) does not have any significant effect during the coupling reaction. These results were also similar to those of Table 3.1 implying good reproducibility of results.

3.4.3 Photomodulation of SP-HRP

3.4.3.1 Introduction

This section studies photomodulation of the modified enzyme (SP-HRP). The reaction is in predominately acqueous solution. The activity of the modified enzyme was observed using 3,3',5,5'–tetramethylbenzidine (TMB) as an enzyme substrate. TMB reacts with HRP to form a blue coloured product, which can be monitored real-time with a spectrophotometer at a wavelength of 650 nm. The rate of colour formation will be used to monitor the effect of photomodultation. The unmodified (native) HRP reaction with TMB was used as a control.

3.4.3.2 Methodology and Experimentation: Photomodulation of SP-HRP

The concentration of native and modified HRP was 10 ng/ml. HRP was modified with SP-COOH via the EDC/Sulfo-NHS reaction established (Section 3.3.2). The conjugation reaction time varied as was as in Section 3.4.2.4. The resulting dye:protein ratios from the rate of coupling analysis (6:1, 8:1, 9:1 and 10:1) were obtained and subsequently stored in ethanol:HEPES; 1:9, in the dark for 12 hours at 4 °C to allow sufficient equilibrium time for dark merocyanine adaptation. 1 ml of solution (10ng/ml) was placed in a quartz cuvette (ensuring that the sample was not exposed to light). 0.5 ml of TMB solution (prepared by dissolving 1 tablet of TMB in 10 ml of 0.05 M Citric Phosphate per Borate buffer) was added and read at every 0.2 seconds for 2 minutes at 650 nm using a UV-Vis Spectrophotometer. The reaction was observed in triplicates. A repeat of the TMB reaction was observed an was a second batch of modified enzyme solution exposed to white LEDs for 3 minutes and scanned as above for 2 minutes. The same procedure (dark and light TMB reaction) was carried out using native HRP for comparisons. All of the measurements were performed in triplicates.

3.4.3.3 Results: Observed Photomodulation of SP-HRP

Photomodulation of SP-HRP was expressed as optical density (OD) signal versus time. This was observed for the four dye:protein molar ratios from the rate of coupling analysis (6:1, 8:1, 9:1 and 10:1) and in triplicates (Figure 3.3).

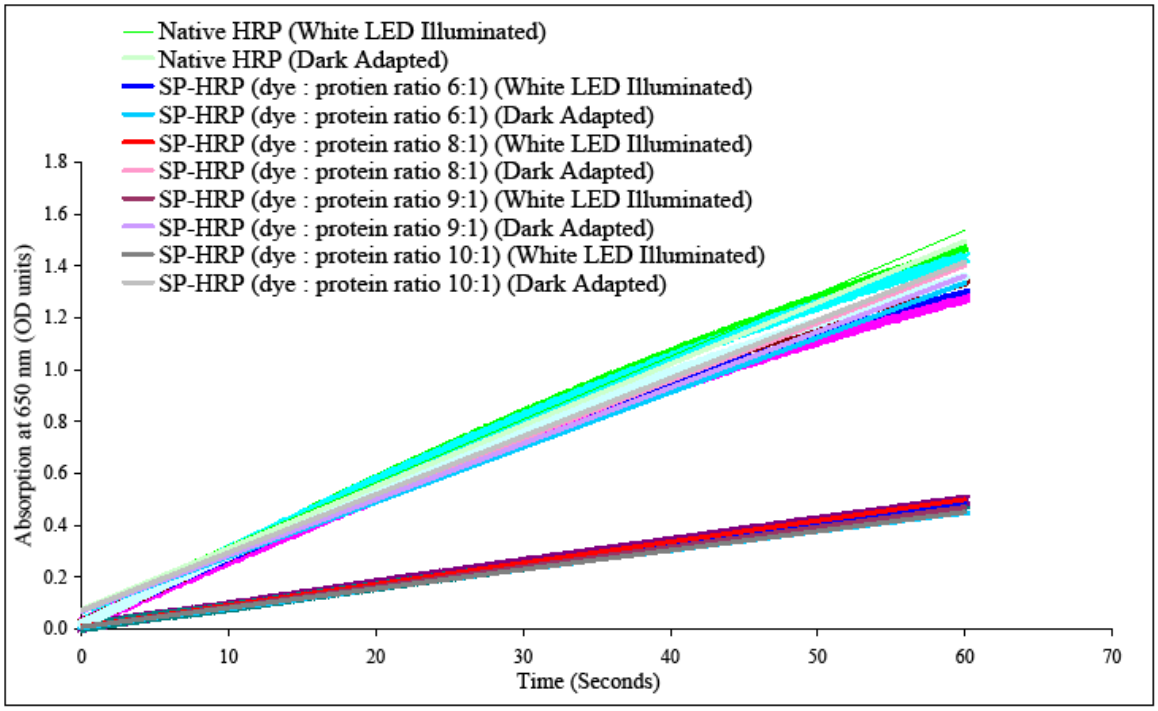


Figure 3.3 : Effect of Illumination on Native and SP-HRP Activity.

TMB reacted with modified and native HRP at concentration of 10 ng/ml. White LED illumination and dark adapted represent the spiropyran form and the merocyanine form of the dye attached to the protein respectively. The data points at 650 nm absorption measurements represent Native HRP and modified HRP (at dye : protein molar ratios of 6:1, 8:1, 9:1 and 10:1). Raw data points were plotted.

The results of Figure 3.3 indicated the native HRP displayed similar activity profile regardless of illumination, therefore implying that the control was valid. The modified HRP (at dye:protein molar ratios of 6:1, 8:1, 9:1 and 10:1) when illuminated with white LED light (to achieve the spiropyran form of the dye) all displayed similar significantly decreased activity; whilst the dark adapted (merocyanine form of the

dye) showed activity almost identical to the native HRP. These results confirm that photomodulation of SP-HRP is feasible and the number of dye attached did not significantly show any difference in results. From these results a linear regression plot was used to calculate the specific enzyme activity of the native and modified HRP under different illuminations (Figure 3.4).

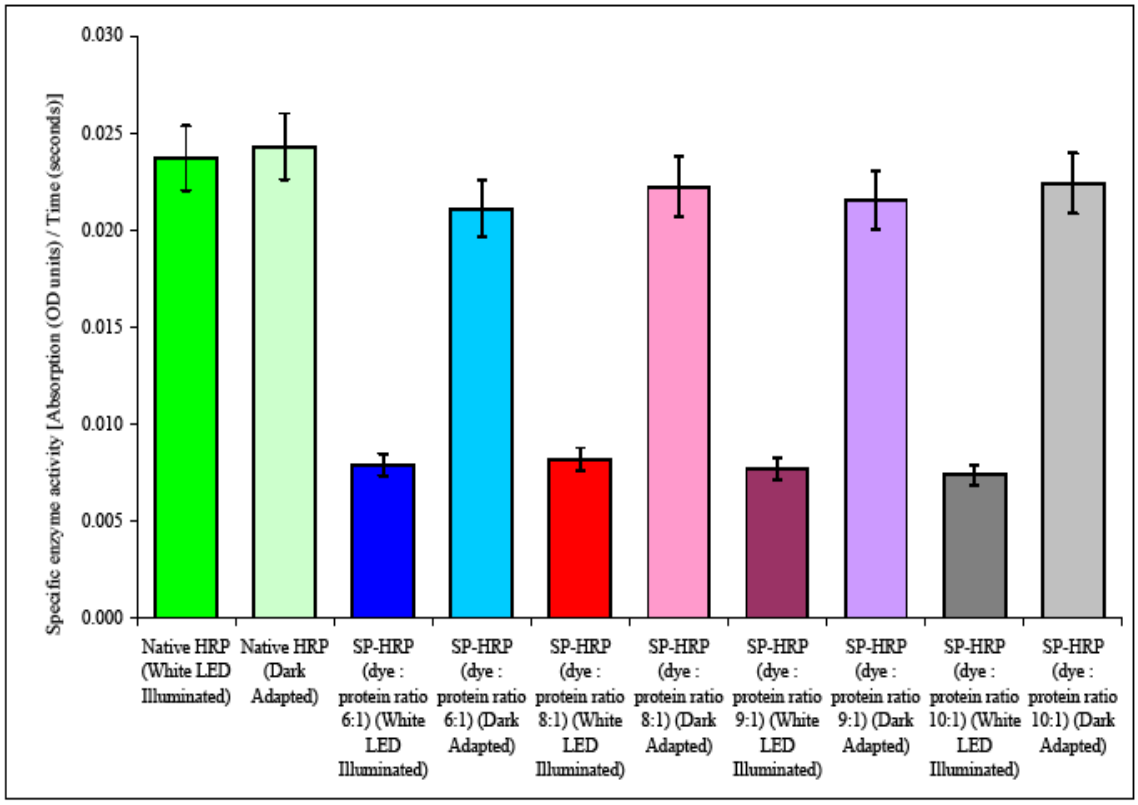


Figure 3.4 : Specific Enzyme Activity of Native and Modified HRP.

Specific enzyme activity of the native and modified HRP protein under two different conditions (visible light and dark adapted). Error bars represent the standard deviation of three repeats.

These results shown in Figure 3.4 indicate that the enzyme activity for the immobilised Native HRP is still conserved. Modified HRP when illuminated with white LED light (to achieve the spiropyran form of the dye) showed ~60 % decrease in specific enzyme activity rate when compared to the dark adapted (merocyanine form of the dye).

3.4.4 Photoswitching of SP-HRP

3.4.4.1 Introduction and Methodology

In order to establish whether the photoswitching properties of the spiropyran dye on the SP-HRP was responsible for photomodulation, the photoswitching properties of the dyes were observed under various illuminations. The correlation between photoswitching and photomodulation was established.

To assess this, SP-HRP was dark adapted for 12 hours at 4 °C to allow sufficient equilibrium time for dark merocyanine adaptation. 1 ml of solution (10ng/ml) was placed in a quartz cuvette (ensuring that the sample was not exposed to light). With appropriate solvents as blanks the SP-HRP solution was observed between 400 and 600 nm using a UV-Vis Spectrophotometer. The solution was then exposed to white LEDs illumination for 3 minutes and then same experiment was observed.

3.4.4.2 Results: Photoswitching of SP-HRP

The photoswitching of SP-HRP was observed in visible white light and in dark adaptation. These include the modified HRP at dye:protein molar ratios of 6:1, 8:1, 9:1 and 10:1. The spectra of the SP-HRP between 400 and 600 nm was observed (Figure 3.5). From theses results, the dye:protein ratio was reflected the in Vis spectra - as the ratio of dye:protein increases, the absorption maxima increases (which indicates the merocyanine concentration).

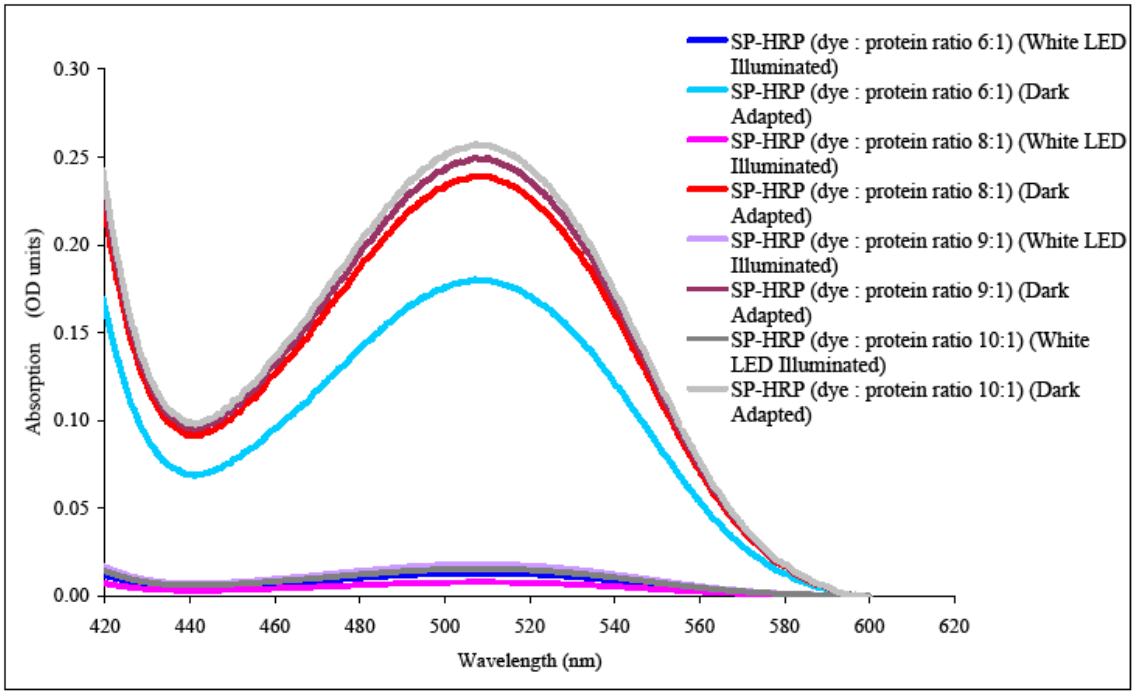


Figure 3.5 : Observed Dye Photoswitching of in SP-HRP.

Photochromism of SP-COOH attached to HRP is represented. The solution was dark adapted to allow merocyanine conversion and observed between 400 and 600 nm. The spiropyran form was also scanned after exposure to white LEDs.

Figure 3.5 shows that relative composition of spiropyran dye on the modified HRP. It can also be established that the change of dye form reflects the modulation observed in Section 3.4.3.3.

3.4.5 Photodegradation (Fatigue) Assessment (SP-HRP: Dye Photoswitching)

To assess whether the coupled dye on SP-HRP was subjected to degradation while repeatedly being changed form one isomer to another, SP-HRP (dye:protein ratio of 10:1) underwent fatigue analysis as in Chapter 2; Section 2.8, whereby the solution mixture was initially put through 10 cycles (of spiropyran / merocyanine conversion) spaced by 12 hours intervals (to allows dark environment conversion to the merocyanine form at 4 °C). The second assessment involved a longer assessment in which SP-HRP was put through 10 cycles spaced by 1 week intervals.

The samples of SP-HRP were initially exposed to the white LED for 3 minutes to ensure spiropyran conversion. 1 ml was placed in a quartz cuvette. Using appropriate solvents as blanks, the SP-HRP solution optical density (OD) measurement was observed at 510 nm. The samples were then placed in a dark environment at 4 °C for 12 hours to allow merocyanine conversion and OD was observed again at 510 nm. The cycle was repeated 9 more times (evenly spaced over 5 days). The second assessment of 10 cycled experiment was conducted just as the first but evenly spaced over 10 weeks. The SP-HRP solution was stored at 4 °C between each cycle. The OD values were plotted against the corresponding switch and graph showing OD and number of switches over time was obtained (Figures 3.6 and 3.7).

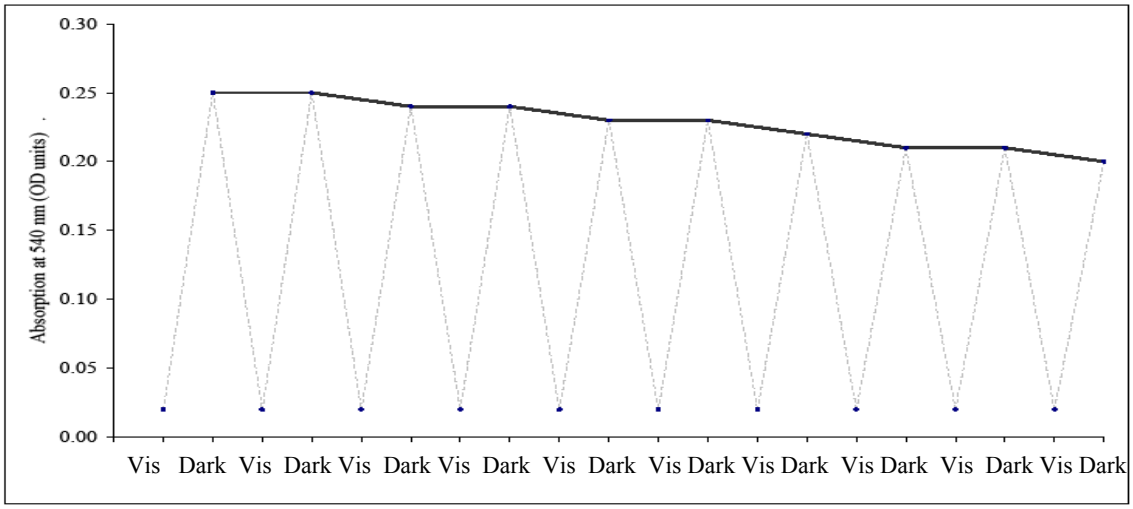


Figure 3.6 : Photodegradation of Dye in SP-HRP Over 10 Cycles (5 Days).

SP-HRP was put through 10 cycles (of spiropyran / merocyanine conversion) spaced by 12 hours intervals (to allow dark adaptation to the merocyanine form at 4 °C) and scanned at 510 nm.

The switching cycles of Figure 3.6 indicates that there is a ~ 25 % gradual degredation of the dye over 10 cycles spread over 5 days. This was observed as the concentration of merocyanine converted in the dark over 12 hours. Spiropyran conversion are more rapid hence these results assume equilibrium is reached during the merocyanine conversion.

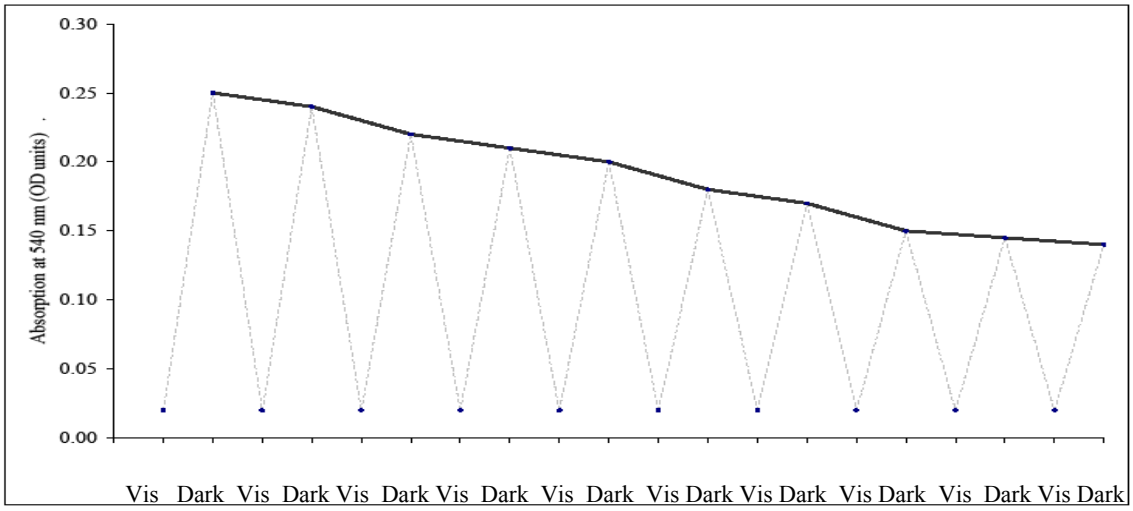


Figure 3.7 : Photodegradation of Dye in SP-HRP Over 10 Cycles (10 Weeks).

SP-HRP was put through 10 cycles (of spiropyran / merocyanine conversion) spaced by 1 week intervals at 4 °C and scanned at 510 nm.

Figure 3.7 indicates that the 10 cycled switch over a longer period of 10 weeks displayes a ~ 40 % degradation. This showed that the dye significantly degraded over the 10 weeks. It implies that the spiropyran dye attached to a protein may not be very stable.

3.4.6 Freeze – Thaw Effect

In order to make sure that the modified protein did not lose activity over time, SP-HRP was stored at 4 °C. However in the attempt to store the dye at a freeze-temperature of -18 °C, it was observed that after 12 hours (and allowed to thaw) the spiropyran dye failed to photoswitch, although the protein remained active. This was confirmed with the same observation over 3 separate SP-HRP samples. The Vis - spectra was observed between 400 and 600 nm (Figure 3.8)

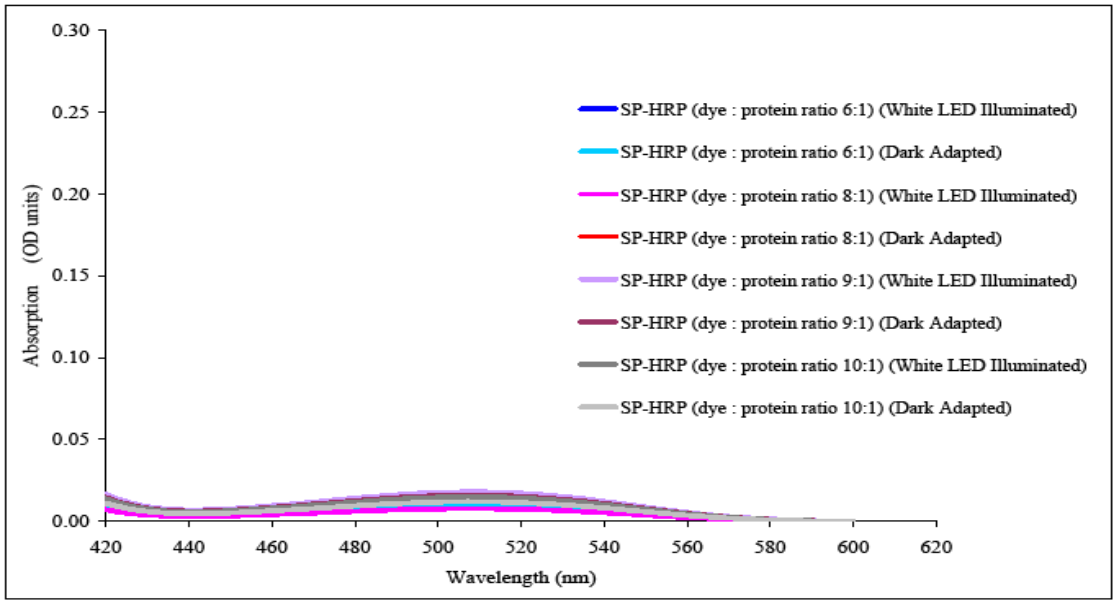


Figure 3.8 : Freeze – Thaw Effect on Photoswitching of SP-HRP.
Photochromism of SP-COOH attached to HRP after storage at -18 °C for 24 hours. The solution was dark adapted and white light illuminated to allow merocyanine and spiropyran conversion respectively and scanned between 400 and 600 nm.

Figure 3.8 shows that the dye remained in the spiropyran form and failed to switch forms regardless of illumination after storage at very low temperature. This effect was briefly highlighted by Yoshida *et al.*, (1994) with the statement "some spiropyrans exhibit irreversible photochromism at very low temperatures" and there has yet to be a formal explanation of this effect.

3.5 Discussion

3.5.1 Conjugation and Characterisation of SP-HRP

The conjugation of SP-HRP via the carbodiimide mediated reaction (EDC/Sulfo-NHS reaction) was successful. The incorporation of polymer bound EDC (P-EDC) was found to simplify the steps, since the dye and the protein were exposed to fewer numbers of chemicals such as 2-mercaptoethanol used to inactivate the EDC. Also the inclusion of the P-EDC eliminates the use of a desalting column to recover the fraction containing the activated protein. P-EDC ensures the by-products of the reaction remain on the polymer and can be isolated by filtration which makes the conjugation process much simpler and cleaner.

Having established photoswitching of the dye in a solution of (1: 9) ethanol: HEPES, the inclusion of this solvent during EDC/Sulfo-NHS conjugation reaction, was known not to interfere with photochromism. With regards to if there was a correlation between the number of dyes attached to the protein and incubation period during the EDC/Sulfo-NHS coupling reaction, it was evidenced that after an hour of reaction, the number of dyes did not increase any further implying that the dye had fully covalently attached itself to all lysine groups that were accessible on the protein.

Non-specific binding was not confirmed to be evident, however since the number of dye attached to the protein did not significantly after magnitude of modulation – it could hypothesised that the dye associates itself to the matrix of the protein and a slight change is affected throught the molecule. HRP is ~ 44 kDa and the dye is about 380 mwt hence the difference in size could facilitate this association and support this statement.

3.5.2 Photomodulation of SP-HRP

Photomodulation of covalently conjugated spiropyran-proteins has been demonstrated a number of times as shown in Chapter 1. The aim of the section was to assess if photomodulation was still observable under the environmental conditions that will

also favour protein activity (*i.e.* antibody activity). Despite loss of UV illumination to switch the dye, in aqueous solution such as HEPES, MES and PBS solution, the dark adaptation was applied in this instance; hence losing the rapidness linked with photochromism. SP-HRP photomodulation has been previously conducted but not under the same environmental conditions defined in this study. Nonetheless, it was observed that photomodulation was still applicable under these conditions and the amount of dye attached to the protein did not significantly affect photomodulation. A plausible explanation is that, the number of dyes needed to affect the binding activity of the protein is probably not significantly high; or that the easily accessible lysine groups were located near the active site of the protein which via direct steric effect or via local distortion of the protein contributed to modulation. Modulation was about 60% when the SP-HRP is allowed to dark adapt (i.e. the dye in the merocyanine form), which is lower than the results published by Weston *et al*., (1999) of 92%; most probably this might be due to the medium in which the study was performed.

The effect of photo induced isomerisation of the spiropyran dye on protein structure has not been fully defined. There have been proposed mechanisms such as structural changes in the protein backbone that would affect the binding properties of the enzyme toward the substrate Willner *et al.*, (1991). There have been other proposals implying photoregulation activity of such proteins may have been attributed to alterations in hydrophilicity-hydrophobicity of the protein microenvironments resulting from the neutral zwitterionic photochromic forms of spiropyrans.

With regards to the freeze – thaw effect, deactivation the dye's photochromic nature on SP-HRP when stored at -18 °C for 24 hours may have been due to the dye attempting to convert to its most thermodynamically stable form at such low temperature, and in the process this might have interfered with the intermolecular interactions of the deposited SP-COOH on the protein. The study was carried out also on an un-conjugated dye and the same effect was recognised. Hence, this possibly confirms a limitation with spiropyran photoswitching at very low temperatures.

3.5.3 Photodegradation (Fatigue) Assessment (SP-HRP: Dye Photoswitching)

Fatigue assessment of the SP-HRP compound was carried out to investigate whether the carbodiimide mediated reaction affected the spiropyran dye after the coupling reaction and also to determine if the covalently attached dye to the protein influences the photoswitching and stability of the dye. Photodegradation of the synthesised dye solely in ethanol: HEPES (1: 9) at pH 7 (section 2.9.4) indicated that the dye was not significantly subjected to fatigue after 10 completed cycles or following 5 weeks of storage. Photoswitching fatigue of the dye in SP-HRP showed 8% degradation after 10 completed cycles without rest intervals (apart from the 12 hours dark adaptation period). Extending the 10 cycle study to over 10 weeks however displayed a 56% degradation of the dye photoswitching capabilities. This stability assessment has not been reported by other groups. The general observation showed that after the coupling of dye the protein, the level of degradation slightly increased, possibly due to the reaction conditions and presence of the protein group on the dye. However due the dark adaption period of 12 hours which extended the study period to 5 days, this observation could have influenced the results since the extended 10 weeks stability assay showed significant fatigue of the dye.

3.6 Summary of HRP Photomodulation

HRP Photomodulation studied in this section re-comfirmed the finding; the purpose of this was to establish the protocol to employ when attempting to photomodulate antibodies and the results attained indicated that the conditions (i.e aqueous buffer medium: ethanol at a ratio of 9:1, conjugation reaction, and characterisation steps) may be regarded favourable to photochromic antibodies.

Chapter 4

PHOTOMODULATION OF ANTIBODY BINDING ACTIVITY MODIFIED WITH SPIROPYRAN DYES

4 Photomodulation of Antibody Binding Activity Modified with Spiropyran Dyes

4.1 Introduction

Photomodulation of antibody binding activity has yet to be successfully demonstrated. The study within this chapter attempts to investigate the feasibility of antibody affinity photomodulation. The study concerns the conjugation of 5 different types of antibodies to SP-COOH (based on the methods developed in Chapter 3), and photomodulation of the spiropyran modified antibodies.

Antibodies usually function well in aqueous solutions and as established from the pervious chapters (Chapters 2 and 3 – the solvent effect on spiropyran dyes, and the feasibility of SP-HRP in aqueous solvents), all the related work presented here is in predominantly aqueous solution (ethanol:HEPES; 1:9). Antibodies are specific bioactive molecules that are extensively applied in commercial systems particularly in biosensing equipment that involved Enzyme-Linked ImmunoSorbent Assay (ELISA) and Surface Plasmon Resonance (SPR). As mentioned in Chapter 1, Section 1.4, regeneration of antibodies in immunoaffinity biosensors is commonly achieved via treatments that lead to loss of antibody activity. The incorporation of a reversible photochromic modulation applied to antibodies to control antigen binding represents an alternative approach over existing methods.

The work presented in Chapter 3 demonstrates a photomodulation system (SP-HRP) in an aqueous system. The degree of photomodulation of enzyme activity (~ 60 %) in these environmental conditions implies that the technique presented in Chapter 3 can possibly be applied to an antibody-antigen system. Although there have been previous attempts to photomodulate antibody-antigen interactions by some research groups (Chapter 1; Section 1.2.4), none have taken this approach. The initial study involves the attempt to modify an antibody fragment of ~ 44 kDa (similar in size to HRP – the reason being this these two protein are of similar size, there maybe some correlation between size of protein and photomodulation), and other whole IgG molecules of ~ 150 kDa, and assess their performance under different illuminations. The antibodies

involved in this study are: a recombinant scAb Anti Atrazine Fragment, Polyclonal Anti GroEL, Polyclonal Anti Phytanic Acid, Monoclonal Anti Staphylococcus Aureus and Monoclonal Anti FITC (Fluorescein Isothiocyanate).

4.2 Chemicals, Biological Compounds, Materials and Equipment

The following biological compounds comsumables were purchased from Abcam, UK: Mouse Anti-FITC (Fluorescein) Monoclonal Antibody, (Unconjugated) (Abcam code: ab2327), Albumin, Fluorescein Isothiocyanate Conjugate Bovine (FITC-albumin) (Abcam code: ab47846), Phytanic Acid - BSA Conjugate (Abcam Code: ab51309), (Rabbit) UV-inactivated *Staphylococcus Aureus* cells (mouse monoclonal IgG, kappa light chain) (Abcam code: ab20002). Donkey Anti-mouse Secondary Antibody IgG-HRP (Cruz marker compatible secondary antibody) (Autogen Bioclear code: Sc-2318) was purchased from Autogen Bioclear and Polyclonal Goat Anti-rabbit Fc - HRP labelled (Stratech Scientific code: NB7179) from Stratech Scientific. BSA-Atrazine recombinant scAb was required from the University of Aberdeen (Haptogen).
The following comsumables were purchased from Sigma Aldrich, UK: Goat Anti-Rabbit IgG (whole molecule)–Peroxidase Antibody (Sigma Code: A6154), Anti-Mouse IgG (whole molecule)–Peroxidase Antibody Produced in Sheep (Sigma Code: A6782), Anti-Human Kappa Light Chains (Bound and Free)-Peroxidase Antibody Produced in Goat (Sigma Code: A7164), Atrazine (Sigma Code: 45330), GroEL (Sigma Code: C7688), Staphylococcus Aureus cell Suspension (Sigma Code: S2014), Purified Recombinant GroEL produced in E. Coli. (Sigma Code: G6532), N-Hydroxysulfosuccinimide Sodium Salt (Sigma Code: 56485), 1-(3-Dimethylaminopropyl)-3-Ethylcarbodiimide, Polymer-Bound (Sigma Code: 424331), Phosphate Buffered Saline (Sigma Code: P4417), HEPES Buffer Salts (Sigma Code: H7523), 3,3',5,5'–Tetramethylbenzidine (TMB) Tablets (Sigma Code: T 5525). Pierce Fluoraldehyde™ Reagent Solution (Pierce code: 26025) was purchased from Pierce, UK. The following were obtained from Fischer Scientific, UK: Centrifugal Concentrator Millipore Microcon YM-10 green 10,000 NMWL 0.5mL (Fischer Code: Fdr-561-030u) and Syringe Filter Non Sterile SFCA membrane green 25mm diameter 0.45μm pore size NALGENE (Fischer Code: Fdm-345-020k). LEDs were acquired from Roithner Laser Technik, Austria; White LEDs, based on GaN 27-33 Cd

(Roithner Laser Technik Code: 5w4hca-H20-Ultra). UV-Vis spectra and Protein assays were recorded on UV-Vis Spectrophotometer UV-2100 (Shimadzu), and Cary Eclipse Fluorescence Spectrophotometer (Varian) respectively. The ELISA plate was read off with Lab Systems iEMS Reader MF microtitre plate reader.

4.3 Covalent Coupling of Spiropyran Dye to Antibody via EDC/Sulfo-NHS Reaction

4.3.1 Introduction

Conjugation was just as for SP-HRP (Chapter 3: Section 3.3.2), however prior to conjugation of the carboxylated spiropyran dye and antibody, competitive binding Enzyme-Linked ImmunoSorbent Assay (ELISA) was performed on each antibody with a known amount of antigen (ranging from 1µg/ml to 10µg/ml) to determine the IC_{50} (inhibitory concentration 50%, Figure 4.2) Figure 4.1 displays the colouration change in an ELISA – the blue colouration indicates the binding signal intensity when the immobilised protein reacts with the labelled substrate, the yellow colouration indicates the end of reaction when quenched (stopped) by the addition of dilute sulphuric acid (H_2SO_4).

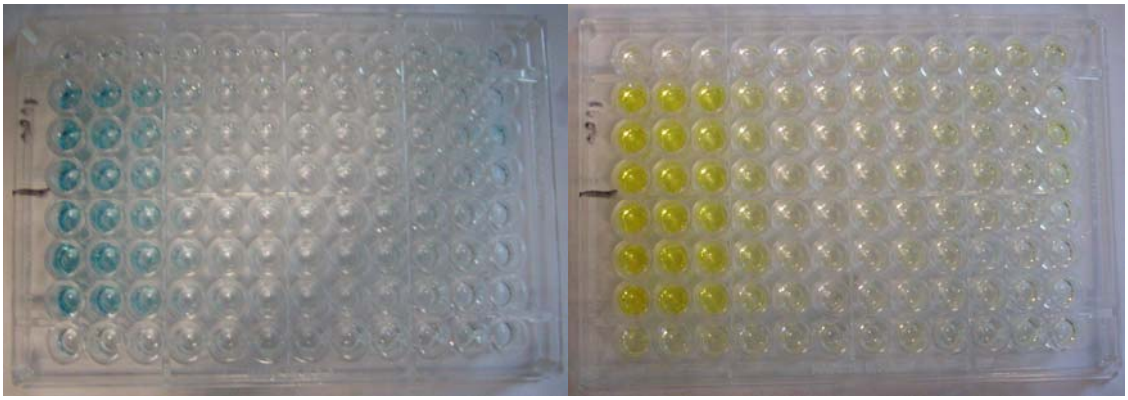


Figure 4.1 : Microtitre Plate During an ELISA.

An illustration of various concentrations of the antibody in an ELISA. End of reaction denoted by the yellow colouration after H_2SO_4 addition.

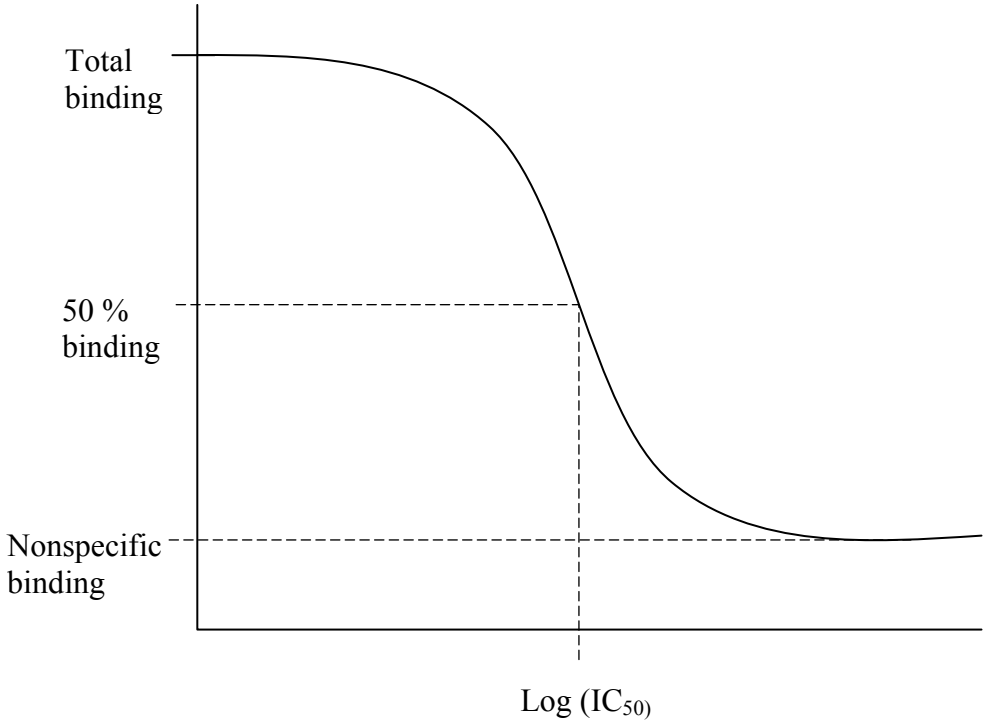


Figure 4.2 : ELISA Competitive Binding Curve.

The top plateau of the curve is equal to antibody completing binding with all available antigen, the bottom of the curve is a plateau equal to nonspecific binding (at low antibody concentration). The concentration of antibody that produces binding half way between the upper and lower plateaus is the IC_{50} (inhibitory concentration 50%) or EC_{50} (effective concentration 50%).

The ELISA carried out for each antibody was via the following protocol:

- Immulon 4 ELISA plate was coated with 100 µl/well of a 1 µg/ml or 10 µg/ml antigen in 1x PBS and Incubate for 1 hour at 20 ˚C.
- The wells emptied by flip-flap motion and blocked with 200 µl 1x PBS containing 1% (w/v) skimmed milk (Marvel) for 1 hour at 20˚C.
- The plate was then washed twice with 200 µl 1x PBS containing 0.1% Tween 20 (PBST) followed by one time 1x PBS.
- 100 µl of antibody at various concentrations was added to the wells in triplicate and incubated for 1 hour at room temperature.
- The plate was washed twice with 200 µl 1x PBS containing 0.1% Tween 20 (PBST) followed by one time 1x PBS.

- The labelled secondary antibody was incubated for an hour at room temperature and washed as before.
- 1 TMB tablet was dissolved in 10ml 0.05 M citric phosphate per borate buffer of which 100 µl was added per well and allowed to develop to desired intensity (within 2 minutes) at room temperature. The reaction was quenched (stopped) with 50 µl/well of 1M H_2SO_4 and read at 450 nm with Lab Systems iEMS Reader MF microtitre plate reader and the IC_{50} was deduced.
- Table 4.1 decirbes in the component for each antibody of study for the ELISA.

Table 4.1 : Antibody with Corresponding Antigen in ELISA.

Table elaborates the antibody and corresponding antigen and secondary antibody involved in current study ELISA.

Immunogen (Antibody)	Antibody Type	Antigen (for coating)	Secondary Antibody
Anti Atrazine Fragment (scAb)	Recombinant scAb	BSA-Atrazine	Anti-Human Kappa Light Chains (Bound And Free) - Peroxidase Antibody Produced In Goat
Purified Recombinant Anti GroEL Produced In E. Coli.	Rabbit Polyclonal IgG	GroEL	Anti-Rabbit IgG (Whole Molecule) - Peroxidase Antibody Produced in Goat
Anti Phytanic Acid – BSA Conjugate	Rabbit Polyclonal IgG	Phytanic Acid - BSA Conjugate	Polyclonal Anti-Rabbit Fc - Peroxidase Antibody Produced in Goat
UV-Inactivated Anti *Staphylococcus Aureus* Cells	Mouse Monoclonal IgG	*Staphylococcus Aureus* Cell Suspension	Anti-Mouse IgG (Whole Molecule) - Peroxidase Antibody Produced in Sheep
Anti IgG FITC - BSA Conjugate, from Rabbit Serum	Mouse Monoclonal IgG	FITC-Albumin	Anti-Mouse IgG - Peroxidase Antibody Produced in Donkey

4.3.2 SP-Ab Coupling

SP-COOH was coupled to each antibody IC_{50} (inhibitory concentration 50%) via the same EDC/Sulfo-NHS involving P-EDC involved with the formation of SP-HRP in section 3.3. EDC and Sulfo-NHS were equilibrated to room temperature. Mass of SP-COOH, based on a equimolar of dye to antibody ratio required as a result of the IC_{50} for each antibody was weighed, dissolved in 1 ml ethanol and made up to a solution of 10 ml 1: 9 (ethanol : 0.1M HEPES at pH 4). 20 mg of Polymer Bound EDC (0.5 mols/g of loading) and 5 mg of Sulfo-NHS (both in excess quantities) were added to the dye solution (in ambient light conditions) and allowed to react for 15 minutes at room temperature (~ 20 °C). The solution was then filtered with a non sterile syringe filter (SFCA membrane, 25 mm diameter 0.45 μm pore size) to remove all by-products from the mixture. The pH of the solution was raised to 7 with NaOH and antibody (at a predetermine volume which measured up to IC_{50} in reaction mix) of each antibody (or antibody fragment) was added and reacted for 1 hour (as noted from the HRP analysis). The solution was passed through a centrifugal concentrator (millipore microcon 10,000 NMWL 0.5 mL) which has a sieve cut-off of 10 kDa. The concentrator membrane was washed with a solution of 1: 9 (ethanol : PBS) at pH 7 to collect the dye labelled HRP.

4.3.2.1 Merocyanine and Spiropyran Effect on Coupling Reaction

Despite no obvious effect being noted with the merocyanine form and spiropyran form effect during the SP-HRP coupling reaction, this was once investigated on the grounds of Sisido *et al*., (1998) observation: that in an experiment involving azobenzene and antibody, the state of the dye (i.e. trans or cis) influenced the photochromic dye binding to antibody. The conjugation was carried out to ensure that dye was in spiropyran form or the merocyanine form as in the HRP study.

4.4 Characterisation of SP-Ab

4.4.1 SP-COOH : Ab Ratio

After coupling of the dye to the protein, the protein characterisation was conducted via Pierce Fluoraldehyde^{TM} protein/peptide assay as in the HRP study. The SP-Ab solution samples were all analysed in the spiropyran form by being initially exposed to white LEDs for 30 seconds. 50µl of modified protein sample plus 50µl of Pierce Fluoraldehyde^{TM} reagent (1:1 ratio) were pipetted into a translucent microtitre plate. The content were mixed for 5 minutes and read on the Cary Eclipse Fluorescence Spectrophotometer after being excited at 340 nm and emission spectra between 450 and 460 nm.

Dye concentration of the modified protein was also analysed by ensuring the solution was in the merocyanine form (*i.e.* pre-stored in a dark environment at 4 ° C for 12 hours) and observed between 400 and 600 nm using a UV-Vis Spectrophotometer. The OD maximum were used to calculate the molar concentration of the dye coupled to the protien with the molar extinction coefficient (ε) of 4 x 10^4 M^{-1} cm^{-1} (at 510 nm in ethanol: PBS (1: 9)).

Having determined the molar concentrations of the protein and the dye of the dye-protein complex (results not shown), the molar ratio of these were calculated and used to define the molar relationship between the spiropyran dye and the antibodies – Tables 4.2 and 4.3. The ratio of the number of dye molecules attached per antibody molecule during conjugation reactions was determined to *(i)* see if the specific nature of individual antibody preparations influence the number of attached dyes and *(ii)* to investigate if the isomeric form of the dye during the conjugation reaction had any influence on the number of dyes attached and / or the subsequent ability of the resultant conjugate to demonstrate antibody binding photomodulation.

Table 4.2 : Ab : SP-COOH Ratio.

Effect of dye's isomer state (merocyanine and spiropyran effect) on the carbodiimide-mediated reaction as well as the ratio of SP-COOH to Ab expressed as the result of molar ratio of protein to immobilised dye after 60 minutes of reaction.

Dye's Isomer State	Immunogen (Antibody) (Ab)	Ab : SP-COOH (molar ratio)
Spiropyran	SP-A (Modified Anti Atrazine, scAb)	1: 6.0 (± 0.4)
	SP-G (Modified Anti GroEL)	1: 8.9 (± 0.4)
	SP-P (Modified Anti Phytanic Acid, BSA conjugate)	1: 12.2 (± 0.3)
	SP-S (Modified Anti *Staphylococcus Aureus* Cells)	1: 8.4 (± 0.5)
	SP-F (Modified Anti FITC, BSA conjugate)	1: 13.0 (± 0.2)
Merocyanine	SP-A (Modified Anti Atrazine, scAb)	1: 6.7 (± 0.2)
	SP-G (Modified Anti GroEL)	1: 10.1 (± 0.3)
	SP-P (Modified Anti Phytanic Acid, BSA conjugate)	1: 11.8 (± 0.1)
	SP-S (Modified Anti *Staphylococcus Aureus* Cells)	1: 8.2 (± 0.1)
	SP-F (Modified Anti FITC, BSA conjugate)	1:12.4 (± 0.3)

The results shown in Table 4.2 indicated that: *(i)* for whole IgG molecules (MW ~ 150 kD) the degree of conjugation ranges from 8.2 to 13 dyes per antibody molecule and for the significantly smaller recombinant antibody fragment (MW ~ 40 kD) a smaller value of 6.0 to 6.7 dyes per antibody fragment and *(ii)* there is no apparent significant difference in the ratio for a given antibody for conjugation performed with the SP-COOH in either the SP or MC isomeric forms.

Table 4.3 : Non-specific Binding Between Ab and SP-COOH.

Non-specific binding was assessed via mixing Ab with SP-COOH under the same conditions as the carbodiimide mediated reaction but without EDC/Sulfo-NHS. Results expressed as the molar ratio of protein to immobilised dye after coupling process.

Immunogen (Antibody) (Ab)	Ab:SP-COOH (molar ratio)
SP-A (Modified Anti Atrazine, scAb)	1: 2.1 (± 0.1)
SP-G (Modified Anti GroEL)	1: 2.8 (± 0.1)
SP-P (Modified Anti Phytanic Acid, BSA conjugate)	1: 3.1 (± 0.1)
SP-S (Modified Anti *Staphylococcus Aureus* Cells)	1: 2.7 (± 0.1)
SP-F (Modified Anti FITC, BSA conjugate)	1: 2.2 (± 0.1)

Table 4.3 indicates that there may be additional background binding signal with the method employed, however it was conluded that, the despite these adjustments, the initial results in Table 4.2 would be referenced through out this chapter, however the results in Table 4.3 will be presented within this thesis should future work clarify these observation.

4.4.2 Freeze – Thaw Effect

Some antibodies are stored at – 18 °C to decrease their rate of denaturation hence the sub-zero (0) °C effect phenomenon observed during the SP-HRP investigation was observed on these SP-Ab. The samples (dye-proetin complex) were stored the at a freeze temperature of -18 °C, and after 24 hours the dye photoswitching capabilities were analysed (Figure 4.3).

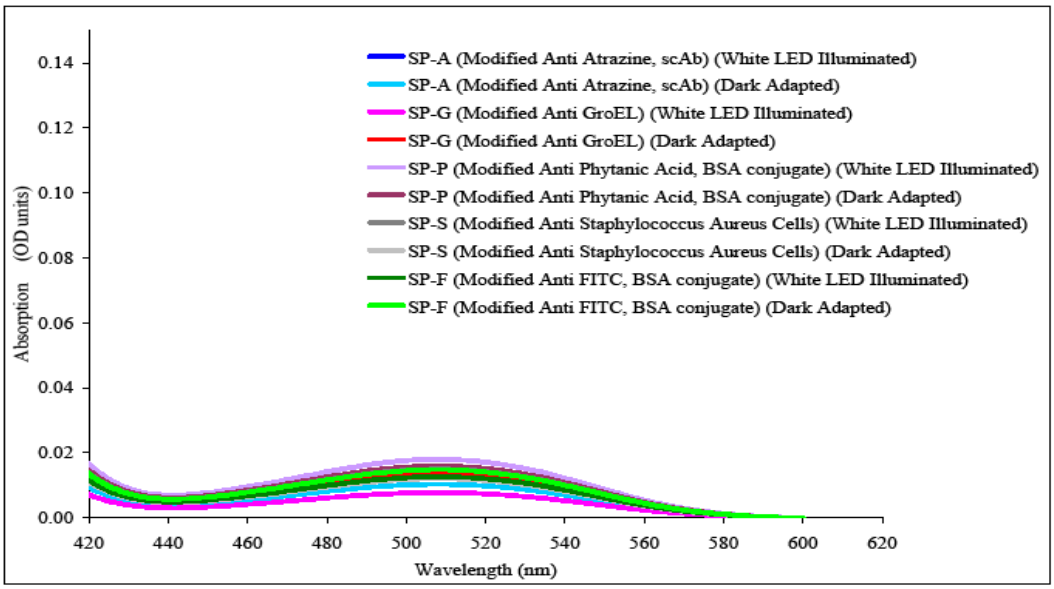


Figure 4.3 : Freeze - Thaw Effect on Photoswitching of SP-Ab.

Photochromism of SP-COOH attached to Ab after storage at -18 °C for 24 hours. The solutions were dark adapted and white light illuminated to allow merocyanine and spiropyran conversion respectively and scanned between 400 and 600 nm.

Figure 4.3 shows similar results of the dye-HRP analysis (Chapter 3, Figure 3.8) that the dye remained in the spiropyran form and failed to switch forms regardless of illumination after storage at very low temperatures. This further re-confirms the observation with the spiropyran modified HRP which also highlights Yoshida *et al.*, (1994) findings that when some spiropyrans are exposed to extreme conditions such as low temperatures exhibit irreversible photochromism.

4.5 Photomodulation via ELISA format of Modified Antibodies

4.5.1 Overview

The conjugated SP-Ab complexes (ten different antibody samples comprising the samples from the conjugation of each of the five antibodies to the SP-COOH in the two different photoisomeric forms) plus as controls, the five native (un-modified) antibody samples were used in an ELISA to determine the antibody binding properties of each of the samples under two different illumination conditions. The

ELISA's were performed under two different illumination conditions and comprised *(i)* with visible illumination using white LEDs and *(ii)* in the dark. Visible illumination resulted in any spiropyran dyes being in the SP isomeric form and placement in the dark resulted in any spiropyran dyes being in the MC isomeric form. To ensure all dyes were in the appropriate isomeric form prior to starting the ELISA's, appropriate pre-exposure to the illumination conditions was performed. The primary objective of the study was to determine if changes in illumination (from visible illumination to removal of illumination – *i.e.* in the dark) would modulate the binding ability of SP-COOH modified antibodies (SP-Ab) using an ELISA. Also to analyse whether the attached photochromic spiropyran dyes had not lost their photoswitching properties after conjugation as well as a verification tool to ascertain that the attached dyes were responsible for modulation, an adsorption visible spectrum was observed between 400 and 600 nm on each SP-Ab under the visible illuminations and after a period of adaptation in the dark. Finally during photochromism of the spiropyran dyes, side reactions can occur during photoisomerisation and as a result, chemical degradation can lead to loss of switching performance over time. To assess whether the conjugated dyes showed any degradation (fatigue) when constantly being switched form one isomer to another, the antibody molecules containing the covalently attached photochromic molecules SP-Ab were initially put through 10 cycles (of spiropyran / merocyanine conversion) spaced by 12 hour intervals (to allow dark adaptation to the merocyanine form). A second assessment, which involved a 10 cycles spaced by 1 week interval was also observed.

Overall the results presented consist of the ELISA of the five antibodies under two the different illuminations - condition *(i)* antibodies modified with the SP isomeric form of the dye during conjugation, - condition *(ii)* antibodies modified with the MC isomeric form of the dye during conjugation, - condition *(iii)* native (un-modified) antibodies observed as controls. Thus, the observed binding signal (optical absorption) obtained at 450 nm after the ELISA - represented the degree of antibody binding of the modified antibodies and native (un-modified) antibodies under the two different illuminations (visible illumination [SP form of the dye] and removal of

illumination – *i.e.* in the dark [MC form of the dye]). Also dye photoswitching profile for each conjugated dye and well as fatigue analysis is presented.

4.5.2 Modified Anti Atrazine Fragment (SP-A)

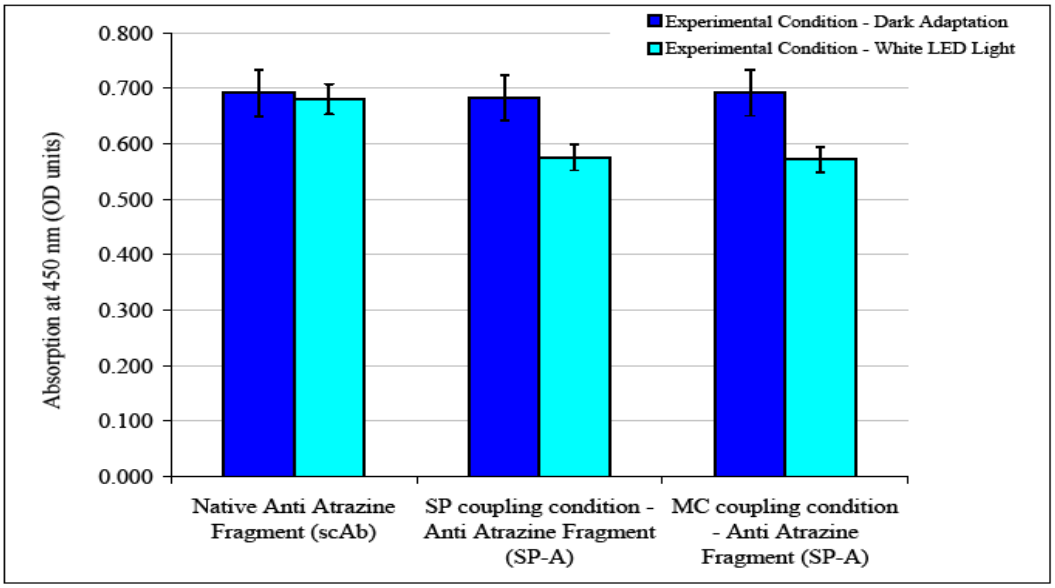


Figure 4.4 : Observed ELISA involving Modified Anti Atrazine Fragment (SP-A)

Native (unmodified) anti Atrazine and modified anti Atrazine were observed in an ELISA under the two different illumination. The extent of antigen-antibody complex formed was a measure of antibody activity. The study was conducted in triplicates and also taking on the board the spiropyran (SP) and merocyanine (MC) coupling conditions.

Figure 4.4 shows that the spiropyran modified anti-atrazine antibody fragments (the only recombinant scAbs in this study) was subject to photomodulation, *i.e.* under condition *(i)* (antibodies modified with the SP isomeric form of the dye during conjugation) - removal of illumination (dark adaptation) during the ELISA displayed similar binding signal as the control (un-modified antibody), whilst ELISA in visible light illumination displayed ~ 15% decrease in antibody binding signal. Similar results were attained under condition *(ii)* (antibodies modified with the MC isomeric form of the dye during conjugation). The native form of the dye as the control functioned as normal regardless of the illumination state. The photochromic nature of

the dye on the antibody fragment was observed (Figure 4.5). Likewise fatigue was observed as illustrated in Figures 4.6 and 4.7.

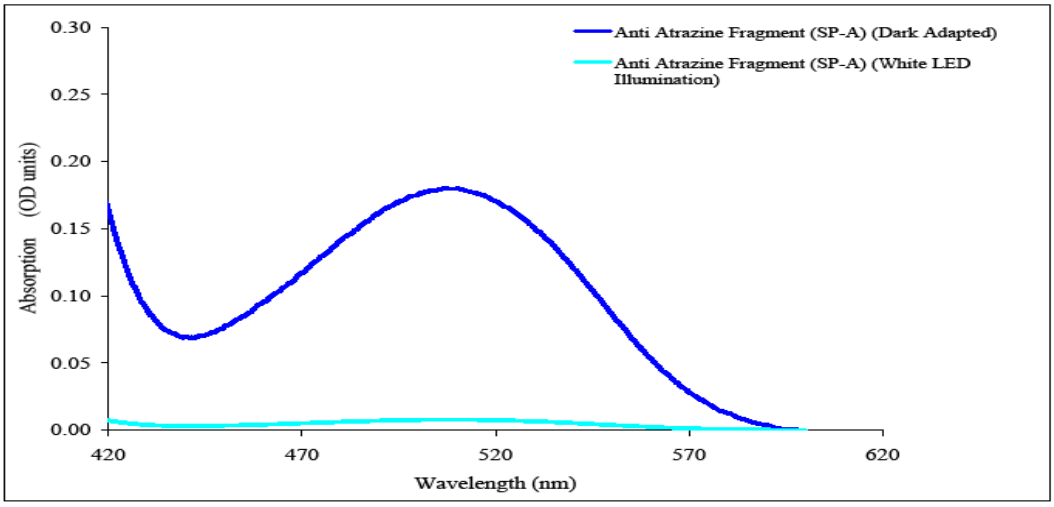


Figure 4.5 : Observed Dye Photoswitching on SP-A.

Photochromism of SP-COOH attached to SP-A. The solution was dark adapted to allow merocyanine conversion and scanned between 400 and 600 nm. The spiropyran form was also scanned after exposure to white LEDs.

Figure 4.5 shows that for SP-A conjugates the photochromic spiropyran dyes retained their photoisomerisation ability and were able to photoisomerise from the coloured MC isomeric form under dark adapted conditions to the colourless SP isomeric form under white LED illumination.

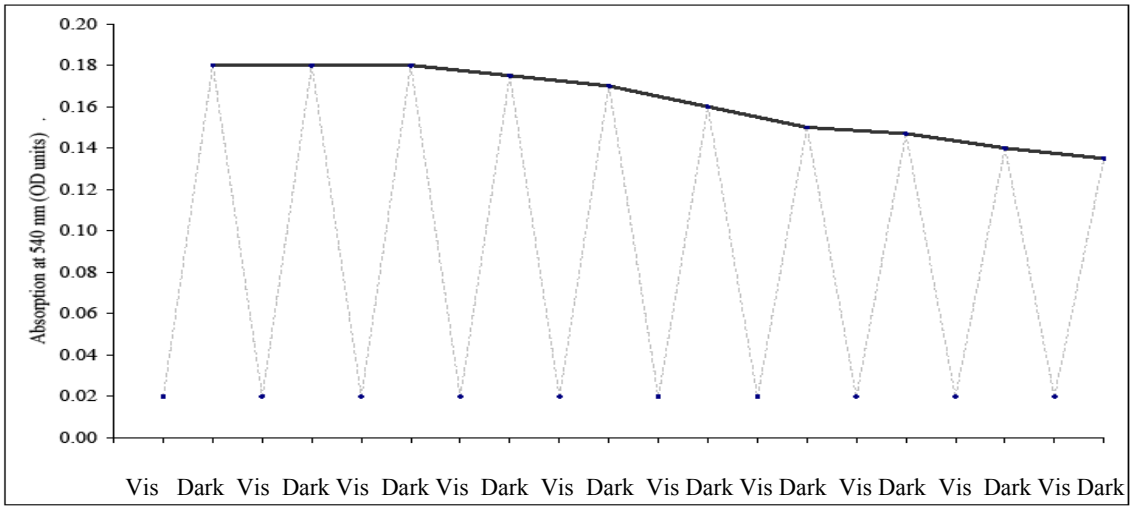


Figure 4.6 : Photodegradation of Dye in SP-A Over 10 Cycles (5 Days).

SP-A was put through 10 cycles (of spiropyran / merocyanine conversion) spaced by 12 hours intervals (to allows dark adaptation to the merocyanine form at 4 °C) and scanned at 510 nm.

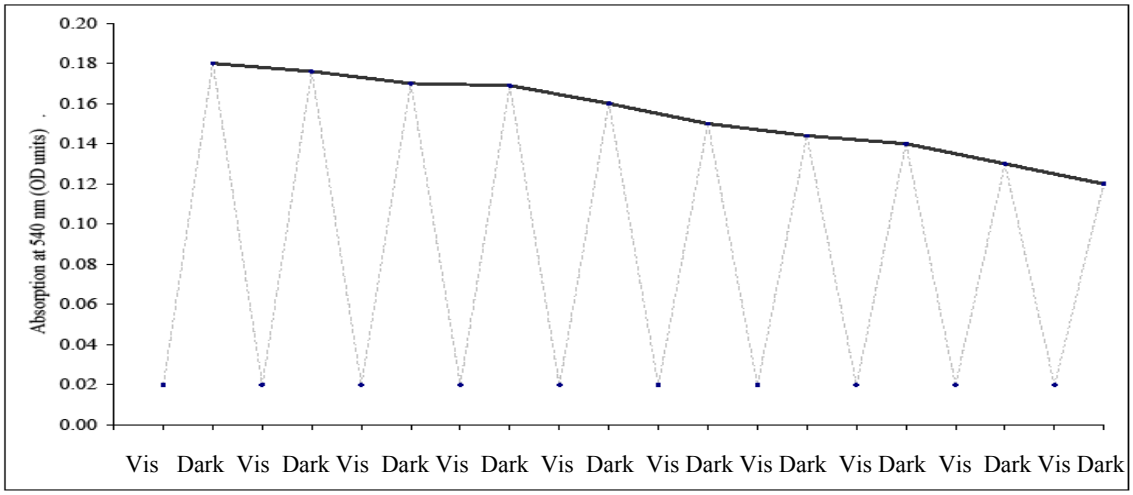


Figure 4.7 : Photodegradation of Dye in SP-A Over 10 Cycles (10 Weeks).

SP-A was put through 10 cycles (of spiropyran / merocyanine conversion) spaced by 1 week intervals at 4 °C and scanned at 510 nm.

The attached dye experiences degradation with time as well when illuminated. The degradation during the 10 weeks was more or less a gradual degradation over time (storage) whereas the immediate switching of dye forms seems to prevent fatigue for the first 4 cycles after which degradation begins.

4.5.3 Modified Anti GroEL (SP-G)

Figure 4.8 shows that the spiropyran modified anti-GroEL antibodies also displayed photomodulation. However, under condition *(i)* (antibodies modified with the SP isomeric form of the dye during conjugation) - removal of illumination (dark adaptation) during the ELISA displayed ~ 46% decrease in antibody binding signal when compared to the binding signal of the control (un-modified antibody), whilst ELISA in visible light illumination displayed ~ 68% decrease in antibody binding signal when compared to the binding signal of the control. Results of condition *(ii)* (antibodies modified with the MC isomeric form of the dye during conjugation) where observed as - removal of illumination (dark adaptation) during the ELISA displayed similar binding signal as the control whilst ELISA in visible light illumination displayed ~ 38% decrease in antibody binding signal when compared to the binding signal of the control. It was also noted that the degree of modulation measured by binding signal difference between the visible illumination and removal of illumination during the ELISA was observed at ~ 40% for both condition *(i)* and condition *(ii)*. This therefore highlights the fact that the interaction between anti GroEL and the dye in its different isomers has a significant effect, prior to the modulation mechanism. The antigen (GroEL) undergoes complex mechanisms, during mediation of protein in cells, it folds involves multiple rounds of binding and encapsulation. It possesses hydrophobic and hydrophilic segments which may have some influence. The photochromic nature of the dye on the antibody fragment was observed (Figure 4.9). Likewise fatigue was observed as illustrated in Figures 4.10 and 4.11.

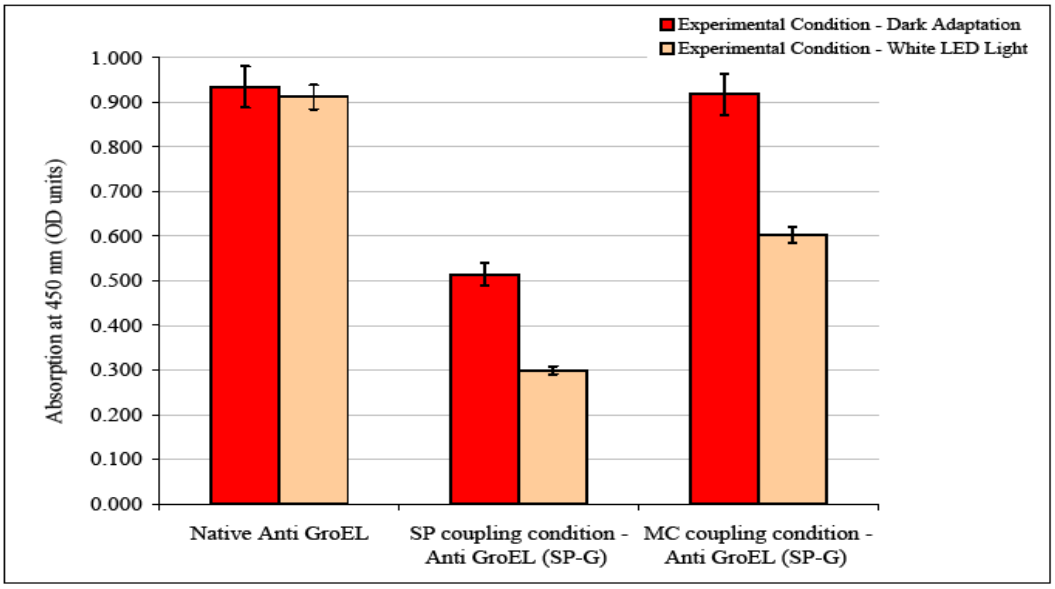


Figure 4.8 : Observed ELISA involving Modified Anti GroEL (SP-G).

Native (unmodified) anti GroEL and modified anti GroEL were observed in an ELISA under the two different illumination. The extent of antigen-antibody complex formed was a measure of antibody activity. The study was conducted in triplicates and also taking on the board the spiropyran (SP) and merocyanine (MC) coupling effect.

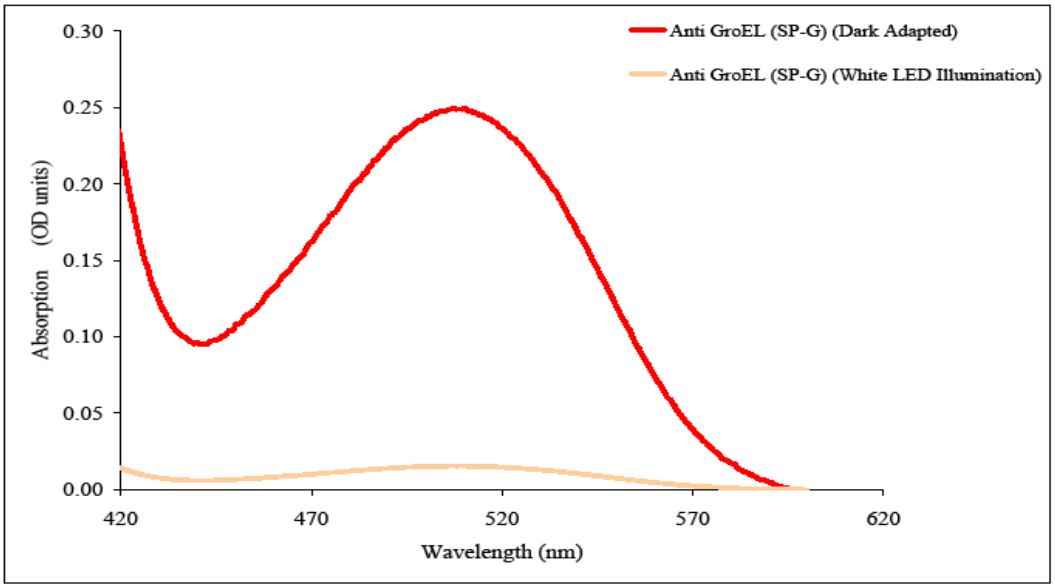


Figure 4.9 : Observed Dye Photoswitching on SP-G.

Photochromism of SP-COOH attached to SP-G. The solution was dark adapted to allow merocyanine conversion and scanned between 400 and 600 nm. The spiropyran form was also scanned after exposure to white LEDs.

Figure 4.9 shows that for SP-G conjugates the photochromic spiropyran dyes retained their photoisomerisation ability and were able to photoisomerise from the coloured MC isomeric form under dark adapted conditions to the colourless SP isomeric form under white LED illumination.

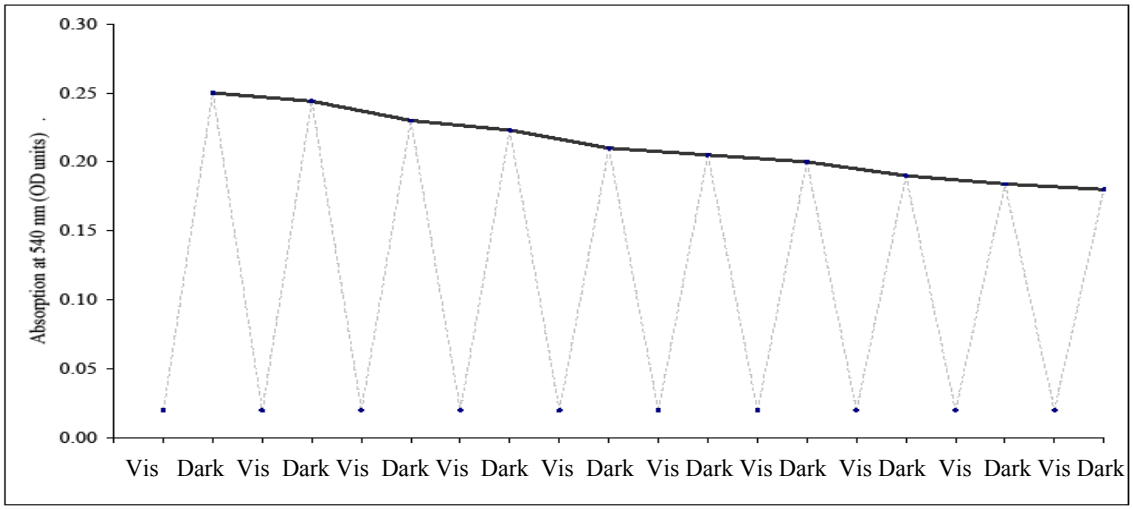


Figure 4.10 : Photodegradation of Dye in SP-G Over 10 Cycles (5 Days).

SP-G was put through 10 cycles (of spiropyran / merocyanine conversion) spaced by 12 hours intervals (to allows dark adaptation to the merocyanine form at 4 °C) and scanned at 510 nm.

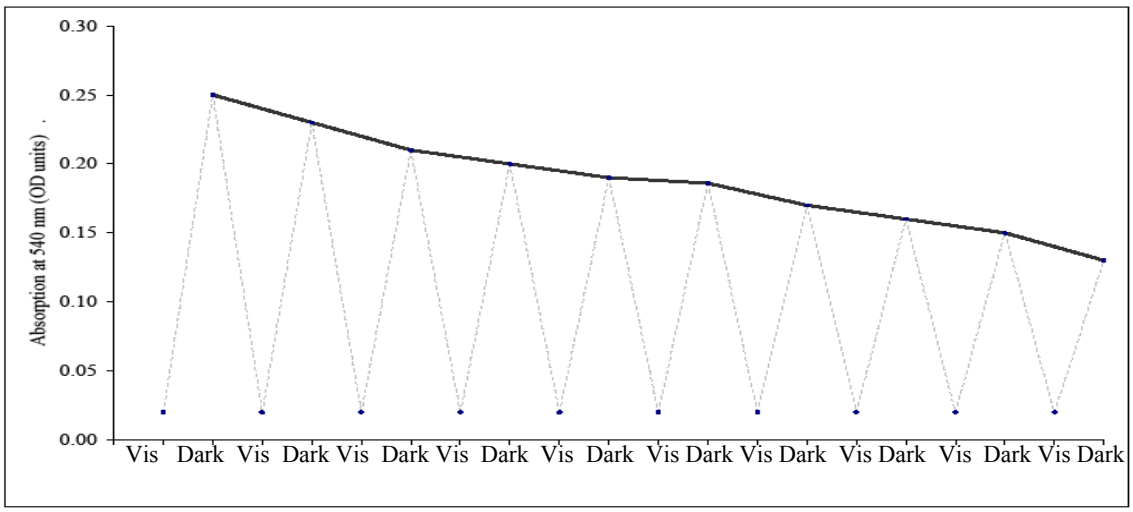


Figure 4.11 : Photodegradation of Dye in SP-G Over 10 Cycles (10 Weeks).

SP-G was put through 10 cycles (of spiropyran / merocyanine conversion) spaced by 1 week intervals at 4 °C and scanned at 510 nm.

The photoisomerisation cycles of Figures 4.10 and 4.11 indicates that there is ~ 40 % gradual degradation of the dye over the 10 cycles spread over 5 days. The 10 cycled isomerisation over a longer period of 10 weeks displayed a ~ 50 % gradual degradation implying that, fatigue was evident under both conditions; however the degree of degradation was 10 % greater with respect to the longer interval period between photoswitching.

4.5.4 Modified Anti Phytanic Acid (SP-P)

Spiropyran modified anti-phytanic acid antibodies also displayed photomodulation of binding signal. Figure 4.12 shows under condition *(i)* (antibodies modified with the SP isomeric form of the dye during conjugation) - removal of illumination (dark adaptation) during the ELISA displayed ~ 63% decrease in antibody binding signal when compared to the binding signal of the control, whilst ELISA in visible light illumination displayed similar binding signal as the control. Results of condition *(ii)* (antibodies modified with the MC isomeric form of the dye during conjugation) were as follows - removal of illumination (dark adaptation) during the ELISA displayed ~ 33% decrease in antibody binding signal when compared to the binding signal of the control, whilst ELISA in visible light illumination displayed similar binding signal as the control. These results indicated that, an illumination condition (*e.g.* dark adaptation as observed in SP-A and SP-G) does not only trigger decrease in binding signal as in this example (SP-P), visible light illumination triggers photomodulation. The photochromic nature of the dye on the antibody fragment was observed (Figure 4.13). Likewise fatigue was observed as illustrated in Figures 4.14 and 4.15.

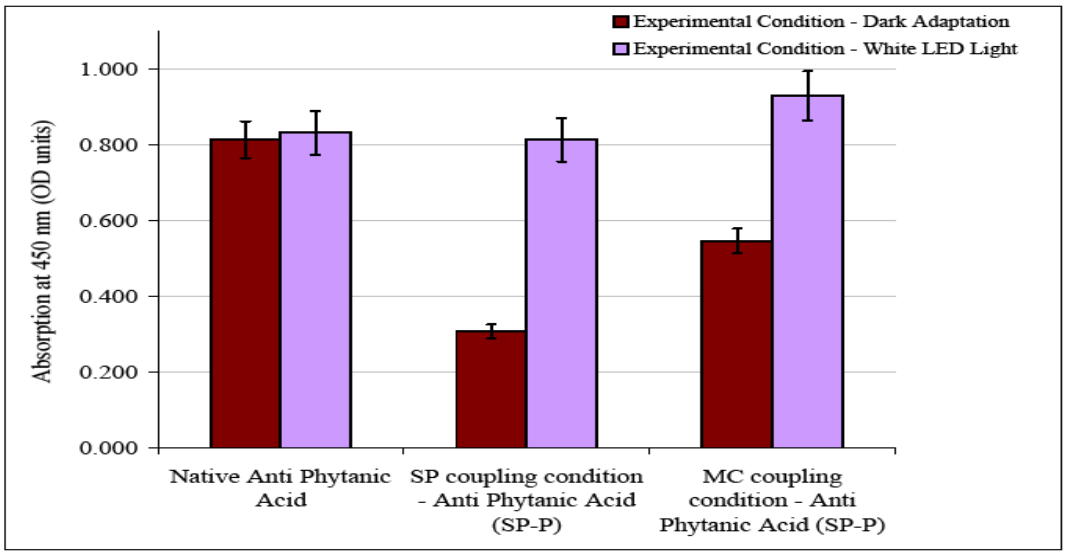


Figure 4.12 : Observed ELISA involving Modified Anti Phytanic Acid (SP-P).

Native (unmodified) anti Phytanic acid and modified anti Phytanic acid were observed in an ELISA under the two different illuminations. The extent of antigen-antibody complex formed was a measure of antibody activity. The study was conducted in triplicates and also taking on the board the spiropyran (SP) and merocyanine (MC) coupling conditions.

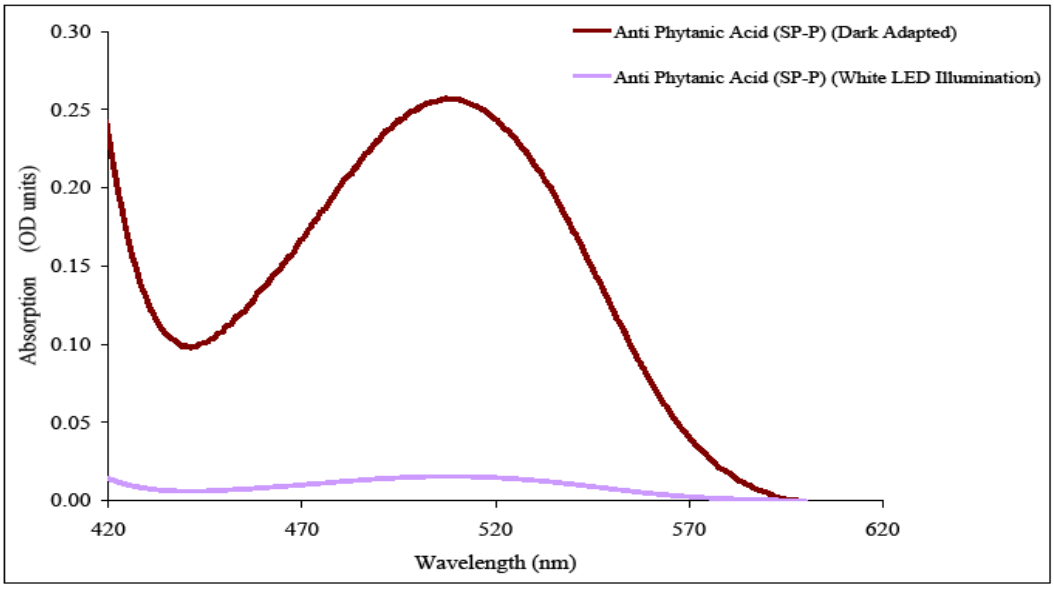


Figure 4.13 : Observed Dye Photoswitching on SP-P.

Photochromism of SP-COOH attached to SP-P. The solution was dark adapted to allow merocyanine conversion and scanned between 400 and 600 nm. The spiropyran form was also scanned after exposure to white LEDs.

Figure 4.13 shows that for SP-P conjugates the photochromic spiropyran dyes retained their photoisomerisation ability and were able to photoisomerise from the coloured MC isomeric form under dark adapted conditions to the colourless SP isomeric form under white LED illumination.

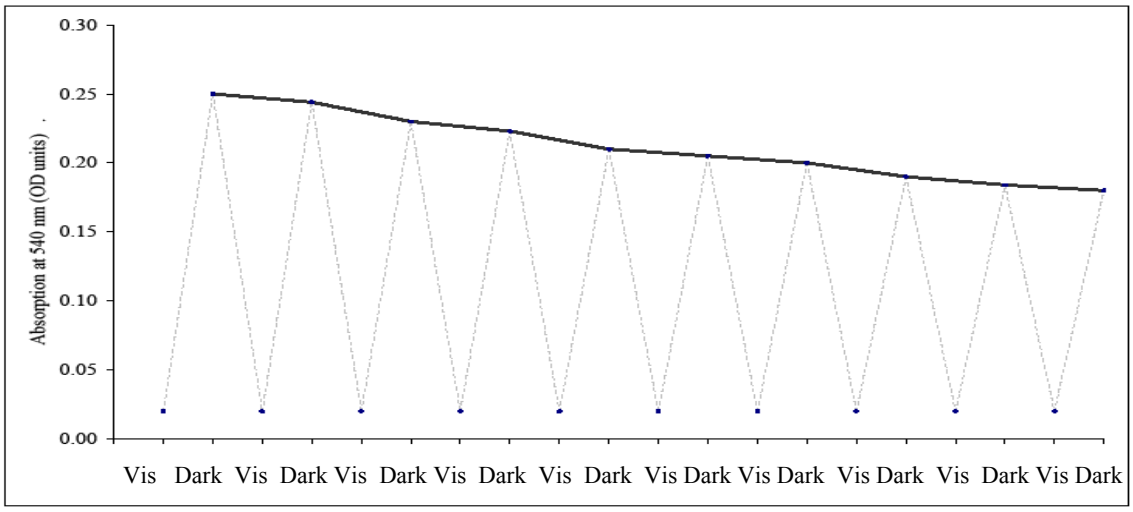


Figure 4.14 : Photodegradation of Dye in SP-P Over 10 Cycles (5 Days).

SP-P was put through 10 cycles (of spiropyran / merocyanine conversion) spaced by 12 hours intervals (to allows dark adaptation to the merocyanine form at 4 °C) and scanned at 510 nm.

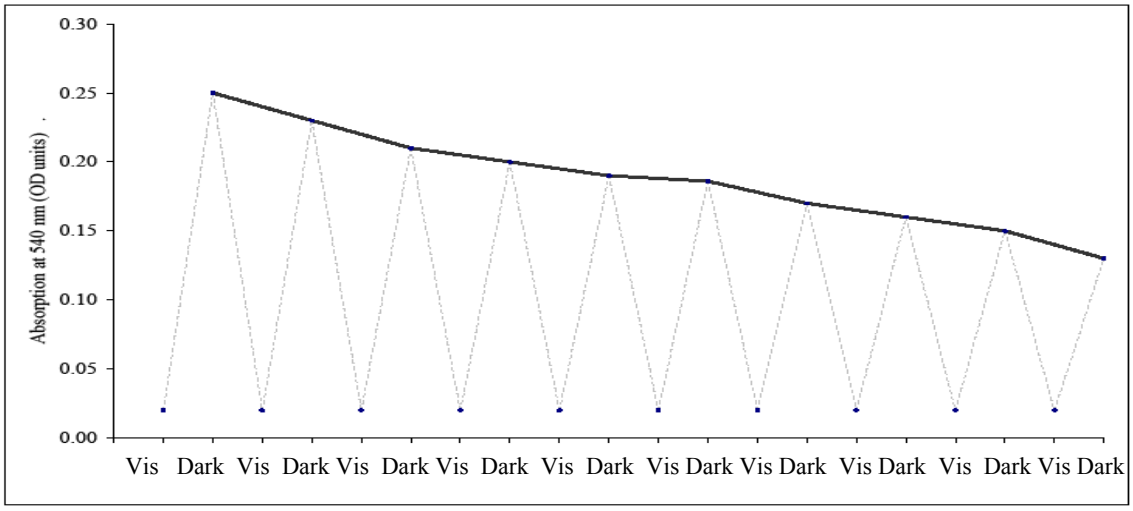


Figure 4.15 : Photodegradation of Dye in SP-P Over 10 Cycles (10 Weeks).

SP-P was put through 10 cycles (of spiropyran / merocyanine conversion) spaced by 1 week intervals at 4 °C and scanned at 510 nm.

The photoisomerisation cycles of Figures 4.14 and 4.15 indicates that there is ~ 35 % gradual degradation of the dye over the 10 cycles spread over 5 days. The 10 cycled isomerisation over a longer period of 10 weeks displayed a ~ 45 % gradual degradation implying that, fatigue was evident under both conditions; however the degree of degradation was 10 % greater with respect to the longer interval period between photoswitching.

4.5.5 Modified Anti Staphylococcus Aureus (SP-S)

Photomodulation of the spiropyran modified anti-*Staphylococcus aureus* (SP-S) indicated the largest degree of modulation within this study (~ 65 % decrease in antibody binding signal) (Figure 4.16). Under condition *(i)* (antibodies modified with the SP isomeric form of the dye during conjugation) - removal of illumination (dark adaptation) during the ELISA displayed similar binding signal as the control (un-modified antibody), whilst ELISA in visible light illumination displayed ~ 70% decrease in antibody binding signal. Similar results were attained under condition *(ii)* (antibodies modified with the MC isomeric form of the dye during conjugation). The photochromic nature of the dye on the antibody fragment was observed (Figure 4.17). Likewise fatigue was observed as illustrated in Figures 4.18 and 4.19.

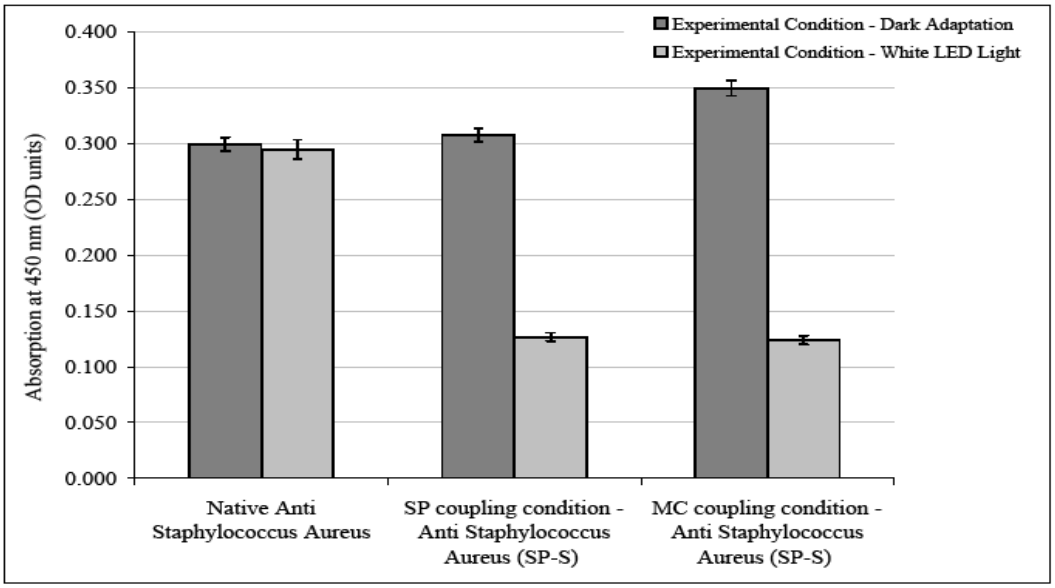


Figure 4.16 : Observed ELISA involving Modified Anti Staph. Aureus (SP-S).

Native (unmodified) anti Staphylococcus aureus and modified anti Staphylococcus aureus were observed in an ELISA under the two different illumination. The extent of antigen-antibody complex formed was a measure of antibody activity. The study was conducted in triplicates and also taking on the board the spiropyran (SP) and merocyanine (MC) coupling conditions.

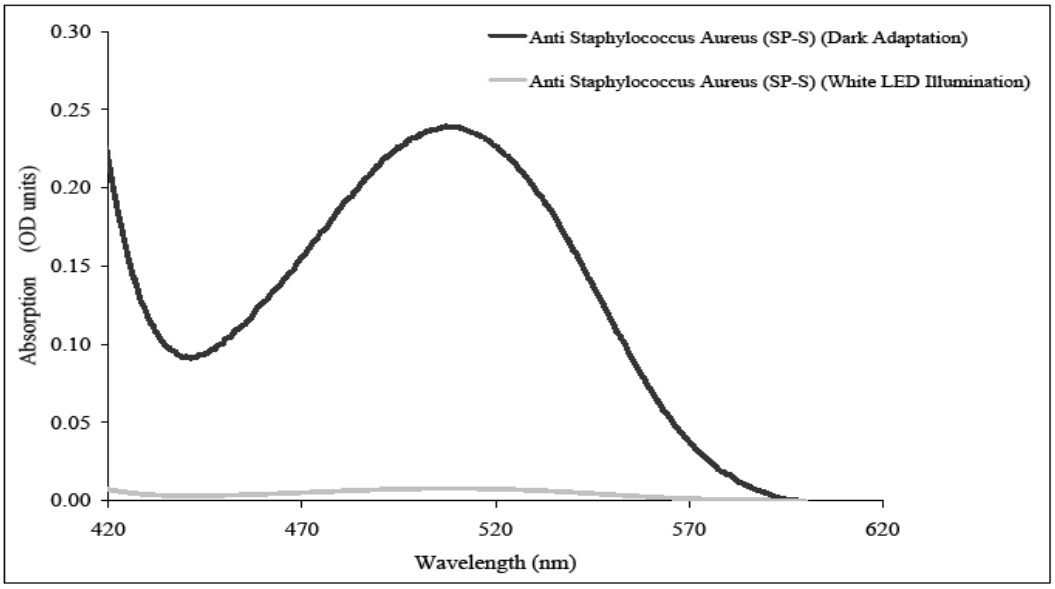


Figure 4.17 : Observed Dye Photoswitching on SP-S.

Photochromism of SP-COOH attached to SP-S. The solution was dark adapted to allow merocyanine conversion and scanned between 400 and 600 nm. The spiropyran form was also scanned after exposure to white LEDs.

Figure 4.17 shows that for SP-S conjugates the photochromic spiropyran dyes retained their photoisomerisation ability and were able to photoisomerise from the coloured MC isomeric form under dark adapted conditions to the colourless SP isomeric form under white LED illumination.

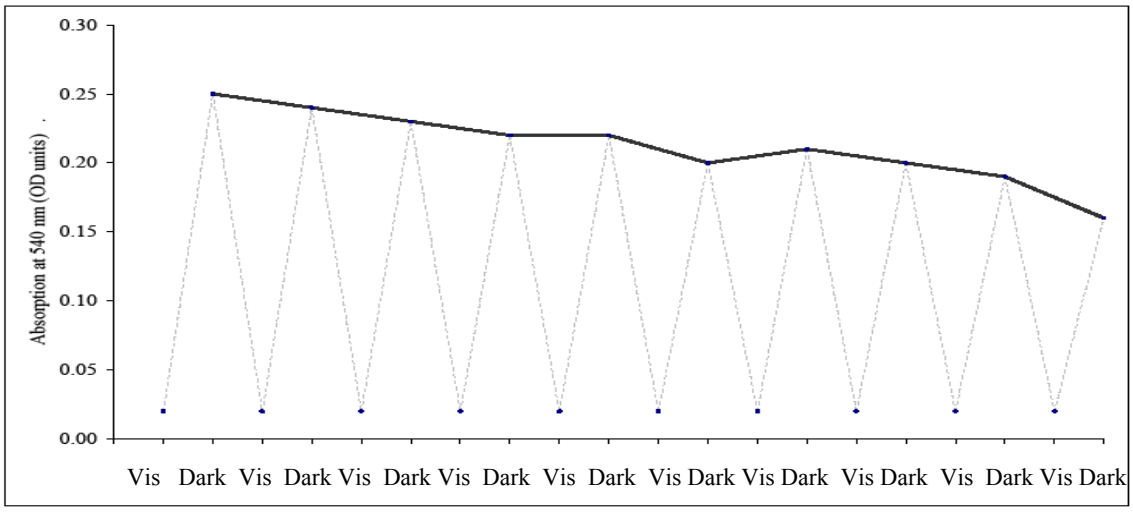


Figure 4.18 : Photodegradation of Dye in SP-S Over 10 Cycles (5 Days).

SP-S was put through 10 cycles (of spiropyran / merocyanine conversion) spaced by 12 hours intervals (to allows dark adaptation to the merocyanine form at 4 °C) and scanned at 510 nm.

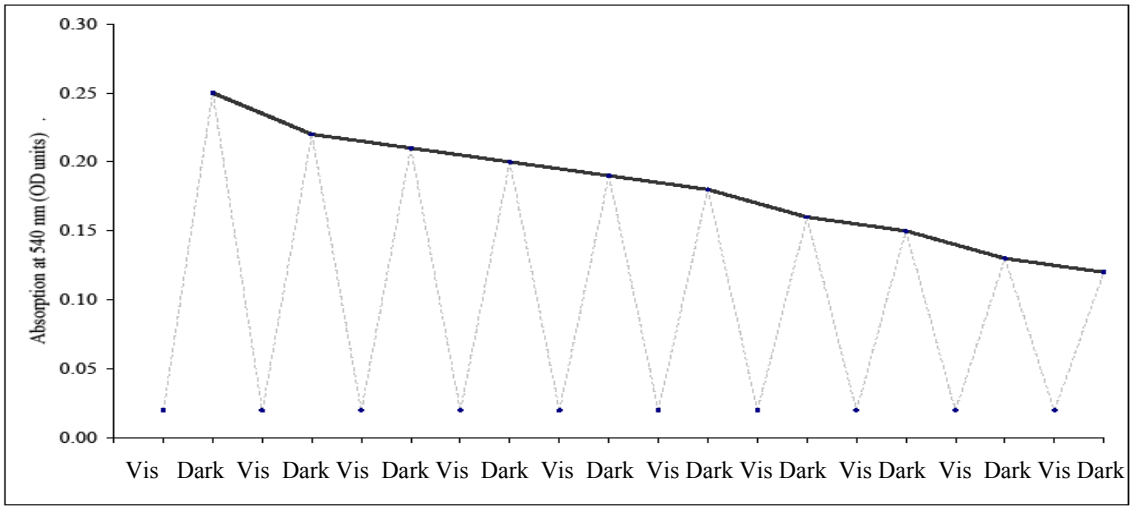


Figure 4.19 : Photodegradation of Dye in SP-S Over 10 Cycles (10 Weeks).

SP-S was put through 10 cycles (of spiropyran / merocyanine conversion) spaced by 1 week intervals at 4 °C and scanned at 510 nm.

The photoisomerisation cycles of Figures 4.18 and 4.19 indicates that there is ~ 40 % gradual degradation of the dye over the 10 cycles spread over 5 days. The 10 cycled isomerisation over a longer period of 10 weeks displayed a ~ 50 % gradual degradation implying that, fatigue was evident under both conditions; however the degree of degradation was 10 % greater with respect to the longer interval period between photoswitching.

4.5.6 Modified Anti FITC (Fluorescein Isothiocyanate) (SP-F)

Figure 4.20 indicated that spiropyran modified anti-FITC antibodies under condition *(i)* (antibodies modified with the SP isomeric form of the dye during conjugation) - removal of illumination (dark adaptation) during the ELISA displayed ~ 55% decrease in antibody binding signal when compared to the binding signal of the control, whilst ELISA in visible light illumination displayed similar binding signal as the control. Similar results were attained under condition *(ii)* (antibodies modified with the MC isomeric form of the dye during conjugation). The photochromic nature of the dye on the antibody fragment was observed (Figure 4.21). Likewise fatigue was observed as illustrated in Figures 4.22 and 4.23.

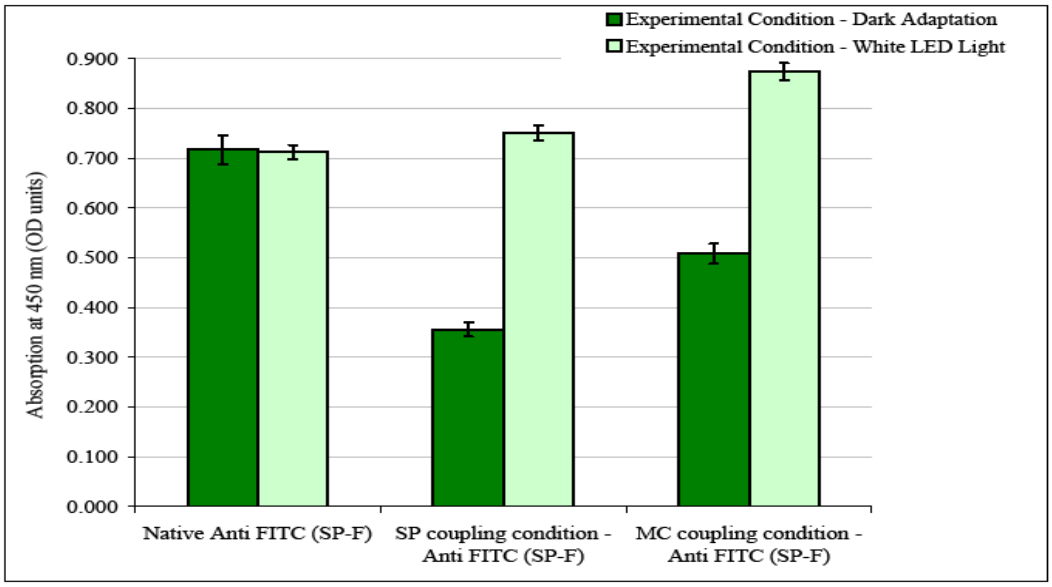


Figure 4.20 : Observed ELISA involving Modified Anti FITC (SP-F).

Native (unmodified) anti FITC and modified anti FITC were observed in an ELISA under the two different illumination. The extent of antigen-antibody complex formed was a measure of antibody activity. The study was conducted in triplicates and also taking on the board the spiropyran (SP) and merocyanine (MC) coupling conditions.

Figure 4.21 shows that for SP-F conjugates the photochromic spiropyran dyes retained their photoisomerisation ability and were able to photoisomerise from the

coloured MC isomeric form under dark adapted conditions to the colourless SP isomeric form under white LED illumination.

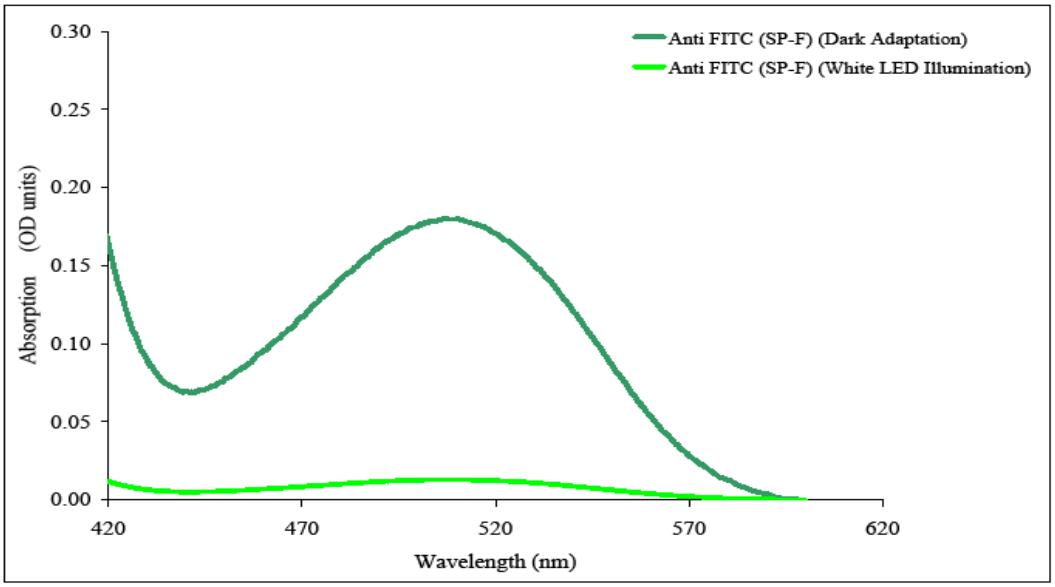


Figure 4.21 : Observed Dye Photoswitching on SP-F.

Photochromism of SP-COOH attached to SP-F. The solution was dark adapted to allow merocyanine conversion and scanned between 400 and 600 nm. The spiropyran form was also scanned after exposure to white LEDs.

The photoisomerisation cycles of Figures 4.22 and 4.23 indicates that there is ~ 20 % gradual degradation of the dye over the 10 cycles spread over 5 days. The 10 cycled isomerisation over a longer period of 10 weeks displayed a ~ 30 % gradual degradation implying that, fatigue was evident under both conditions; however the degree of degradation was 10 % greater with respect to the longer interval period between photoswitching.

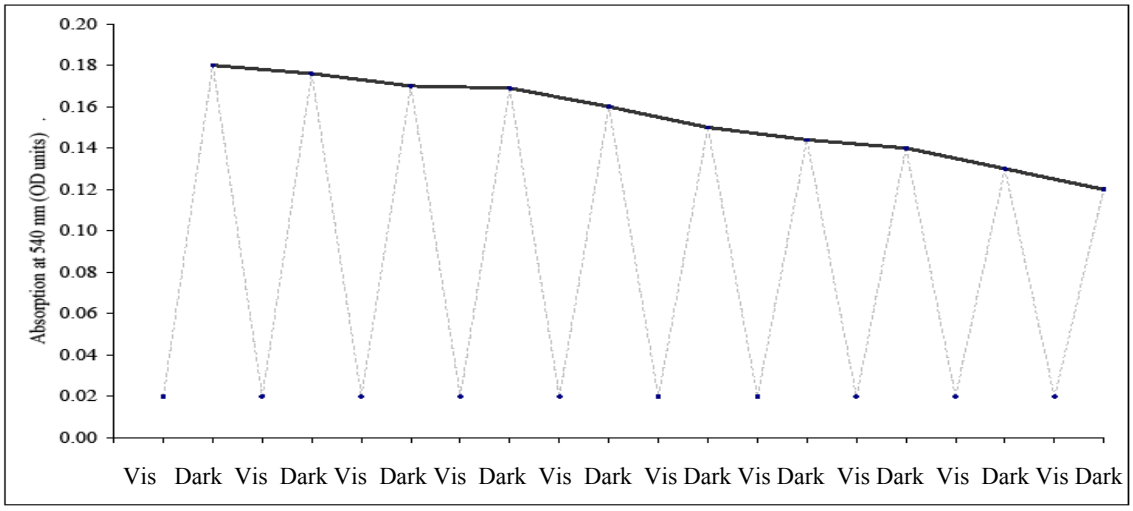


Figure 4.22 : Photodegradation of Dye in SP-F Over 10 Cycles (5 Days).

SP-F was put through 10 cycles (of spiropyran / merocyanine conversion) spaced by 12 hours intervals (to allows dark adaptation to the merocyanine form at 4 °C) and scanned at 510 nm.

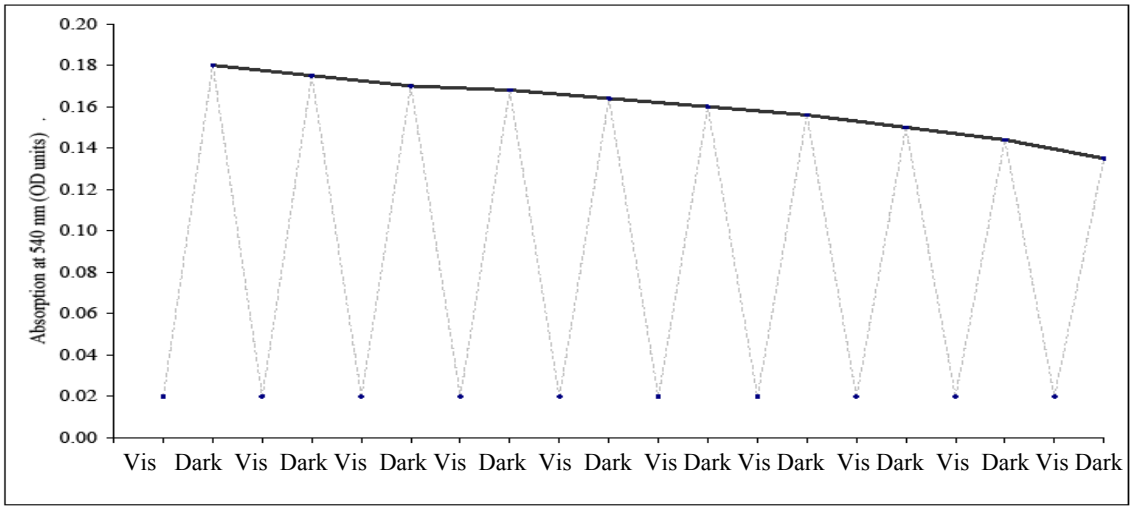


Figure 4.23 : Photodegradation of Dye in SP-F Over 10 Cycles (10 Weeks).

SP-F was put through 10 cycles (of spiropyran / merocyanine conversion) spaced by 1 week intervals at 4 °C and scanned at 510 nm.

4.5.7 Summary of Photomodulation of SP-COOH Modified Antibodies in an ELISA

In summary of the photomodulation analysis shows results of a range of different types of antibody, their degree of modulation and the illumination condition that triggers modulation. One salient observation of the studied modified antibodies is that all underwent photomodulation. However, the degree of modulation varied with each antibody as well as the illumination which triggered modulation.

4.6 Discussion

4.6.1 Conjugation and Characterisation of SP-Ab

The conjugation of spiropyran modified antibodies (SP-Ab) via the carbodiimide-mediated reaction (EDC/Sulfo-NHS reaction) was also successful. However, taking into account that an antibody contains ~ 90 lysine residues (according to entry in structure 1hzh in the RCSB Protein Database), it can be suggested that not all the lysine groups were modified during conjugation as the molar ratios of SP-COOH to Ab reflects this (Table 4.2) – maximum of 13 out of a possible 90 moles of spiropyran dyes were covalently attached to an antibody. This observation may have been as a result of inaccessibility of lysine groups due to their location and/or multiple folded protein nature (tertiary and quaternary structures) of the antibody molecule. In the case of the antibody fragment (anti-atrazine), taking into account its size (~ ¼ of the size of the full IgG molecule), fewer lysine groups were expected to be available for coupling and was confirmed with the lower molar ratio of dye to protein (Table 4.2). These observations also suggests that only a small fraction of the conjugated photochromic spiropyran dyes triggered photomodulation and as such, the number of dyes involved in photomodulation was significantly low.

In the attempt to establish whether the two different dye isomeric forms employed during the conjugation reaction had an effect on the number of dye covalently

attached to the antibodies and its overall effect on the photomodulation study, it was observed that; with respect to number of moles of dyes coupled to the antibodies, there was no significant difference for both conjugation conditions *(i)* and *(ii)*. [*i.e.* condition *(i)* - implies antibodies modified with the SP isomeric form of the dye during conjugation, and condition *(ii)* - implies antibodies modified with the MC isomeric form of the dye during conjugation]. The observation whether the two different dye isomeric forms employed during the conjugation reaction had any effect on the photomodulation study is addressed within the photomodulation of SP-COOH modified antibodies in an ELISA (SP-Ab) discussion section.

Most antibodies require storage below 4 °C, ideally -18 °C (deep freezer temperature) is preferred; however the sub-zero °C effect phenomenon, whereby the spiropyran fails to photoswitch (after it being thawed and rewarmed to room temperature), casts a shadow on further applications with regards to its use after low temperature storage.
The phenomenon experienced could be due to distortion of the intermolecular forces of the dye. Spiropyran dye switching is dependant on the form which is thermodynamically stable as a result of the environmental or illumination conditions; hence the establishment of the frozen solvent may prevent any further changes in the dye form which possibly leads to distortion in its molecular structure in an attempt to aid configuration.

4.6.2 Photomodulation of SP-Ab

Photomodulation of spiropyran modified antibodies in an antibody-antigen binding reaction has not been successfully demonstrated until now - it is reported here for the first time, citing a maximum of ~ 65 % photomodulation of antibody binding signal. The inclusion of various antibody types (single chain fragments, monoclonals and polyclonals) was to establish photomodulation among a diverse assortment of antibodies. The approach was to covalently attach photochromic spiropyran dyes to antibodies with the anticipation of that under appropriate illumination; one of the two photoisomers of the dye will have an effect on (modulate) the binding affinity of the modified antibody.

Anti-atrazine single chain fragments was a key candidate antibody since its molecular size (~ 40 kDa) was very similar to spiropyran modified HRP - a proven photomodulated system established in Chapter 3. It was contemplated that the size of an antibody (~ 150 kDa) may prove to be too large a molecule to photomodulate its binding function, hence a smaller size molecule *i.e.* antibody fragment was anticipated to display a substantial degree of modulation. However, the modified antibody fragment represented the smallest degree of modulation (~ 15 %) whilst the largest modified antibody anti-*Staphylococcus aureus* IgG displayed the largest modulation of ~ 65 %. The degree of modulation may be linked with the number of moles of spiropyran dye attached to the antibodies with the antibody fragment possessing fewer dyes than the whole IgG antibody. Having already established that the overall number of dyes covalently attached to the antibody was significantly low for all antibody types, it can also be contemplated the number of dyes may not be the only factor linked with the degree of modulation but also the position of the dyes on the antibodies (not investigated within the scope of this study). Another factor that may have affected modulation but only evident with the modified anti-GroEL antibody (SP-G) (Figure 4.8) is the photoisomeric state of the spiropyran dye during its conjugation with the antibody. It is known that that SP isomer of the dye is neutrally charged and is hydrophobic, whereas the MC isomer of the dye is a more hydrophilic, zwitterionic compound with delocalised charged electrons. These fundamental molecular differences in chemical properties could imply variation in the mechanism of action, and as such have an effect on the overall modified antibody binding activity.

Concerning the illumination condition responsible for modulation, anti-atrazine fragment, anti-GroEL and anti-*Staphylococcus aureus* were modulated by visible illumination *i.e*. the SP form of the attached dye triggered a reduction in antibody binding signal. The removal of illumination (*i.e*. the MC form of the attached dye) triggered the modulation of anti-phytanic acid and anti-FITC (Table 4.4 – summary of the observed modulation of the antibodies involved in this study). This highlights the fact that generally, photomodulation of modified antibody binding affinity is feasible

but there are variations in the reactive nature of the modified antibodies and ultimately the mode of photomodulation. This observation needs further experimental support to aid understand the mechanism of modulation. For example, examination of the positions of conjugated dye on the antibody before, during and after the photomodulation phenomenon, and the structural changes the antibody undergoes during these conditions.

Table 4.4 : Summary of Antibody Photomodulation within this Study.

Tabular comparison of the mode and degree of photomodulation of the antibodies in this current study.

Immunogen (Antibody)	Antibody Type	Average Degree Of Modulation	Dye Form Responsible For Modulation
Anti Atrazine Fragment (scAb)	Recombinant scAb	~ 15 %	Spiropyran Form
Purified Recombinant Anti GroEL Produced In E. Coli.	Rabbit Polyclonal IgG	~ 40 %	Spiropyran Form
Anti Phytanic Acid – BSA Conjugate	Rabbit Polyclonal IgG	~ 50 %	Merocyanine Form
UV-Inactivated Anti *Staphylococcus Aureus* Cells	Mouse Monoclonal IgG	~ 65 %	Spiropyran Form
Anti IgG FITC - BSA Conjugate, from Rabbit Serum	Mouse Monoclonal IgG	~ 55 %	Merocyanine Form

From the results presented in this chapter, the following hypothesis is proposed: the degree of photomodulation of antibodies varies from antibody to antibody since the mechanism of antigen-antibody complex formation also varies between antibodies. The presence of BSA conjugates on a whole IgG molecule influences the isomeric form of the dye that causes modulation.

4.6.3 Photodegradation (Fatigue) Assessment (SP-Ab : Dye Photoswitching)

Fatigue of the dye was experienced throughout all applications, although the extent of fatigability varied among the various modified antibodies. These results were similar to the fatigue experienced by the spiropyran modified HRP in the previous chapter. The immediate switching seems to be fairly stable during the first 3 to 4 switches followed by a significantly noticeable degradation. This may be because the mode of switching takes on an additional time effect (incubation time for merocyanine conversion), the dye may also be subjected to storage stability effect in the process of immediate switching analysis. The long storage stability test reveals that the dye's performance decreases with the number of weeks stored at 4 °C. This may not be that significant with regards to the intended application – antibodies may not be kept at 4 °C and reused over a 3 month period due to the denaturation occurring in the antibody. In comparison to the unconjugated dye (Chapeter 2), fatigue is negligible. This suggests that fatigue with respect to SP-COOH conjugated to antibodies may be due to: *(i)* the conjugation reaction treatment, and/or *(ii)* the molecular instability of the spiropyran dye after it was coupled to the antibody.

Chapter 5

ANTIBODY-ANTIGEN BINDING AND SPIROPYRAN DYES PHOTOSWITCHING IN IONIC LIQUIDS

5 Antibody-Antigen Binding and Spiropyran Dyes Photoswitching in Ionic Liquids

5.1 Overview

This chapter presents the introduction of antibody-antigen binding in ionic liquids. This innovative study involves the investigation of the feasibility of antibody-antigen binding in 3 different types of ionic liquids within the same class (deep eutectic solvents; choline chloride derivatives). The aim of this study is to use ionic liquids (as candidates for traditional organic solvents replacement) to deliver improvements in the performance of analytical methods. The nature of this investigation is based on a modified ELISA technique, which allowed inclusion of various concentrations of ionic liquids in the assay process. Spiropyran dye photoswitching properties were also investigated, with the intention of observing photomodulation of the modified proteins studied (in Chapters 3 and 4) in ionic liquids.

5.2 Relevance of Ionic Liquids to Current Study

Biomolecules that exhibit binding functions such as antibodies (and their corresponding antigens) find a diverse range of applications in modern biotechnology. It is common practice given that in most cases, the presence of an appropriate solvent *i.e.* water, is required to allow biomolecule function; the removal of water will result in the cessation of biological function. Antibody-antigen binding requires the presence of bulk quantities of water – either with the biomolecules in aqueous solution or associated with surfaces that are in contact with bulk aqueous solutions. The use of biomolecules in applications that require contact with strongly evaporative media will result in the rapid removal of water by evaporation. Conditions where the water content is low or water is evaporated (high gas/liquid surface area to volume ratio) can be regarded as extreme environments for biological function.

Ionic liquids as previously described in Chapter 1, Section 1.6 are a class of materials that have recently attracted a growing interest as replacements for traditional organic solvents in a number of industrial applications. The recent interest surrounding ionic liquids with regards to "green chemistry" has largely been as a result of the fact that ionic liquids have very low vapour pressure (in contrast to traditional solvents), and hence, emit little or no volatile organic compounds. Biomolecules such as enzymes have been studied in ionic liquids and have shown to enhance enzyme activity, stability, and selectivity (Yang *et al.*, 2005). However, application of an antibody-antigen binding technology in ionic liquids has yet to be established. The use of ionic liquids with very low vapour pressure may eliminate instability caused by the rapid evaporation of traditional water based solvents of an antibody-antigen technology in extreme environments. The idea of antibody-antigen technology in applications to detect volatiles, in operation in icy environments relevant to understand life in such conditions, fluid handling and assay in low pressures (*e.g.* operation at Martian atmospheric pressures in planetary applications; *in situ* detection of micro-organisms in the high atmosphere and therefore at low pressure [Sims *et al.*, 2005]), may be feasible with the use of ionic liquids as solvents. Other immediate beneficiaries of the use of ionic liquids in sensor technology includes its potential applications in gas phase sensing, which may include airborne microbial detection.

In view of the fact that ionic liquids applications are generally aimed at improving technologies, the approach within this study is to apply ionic liquids in an antibody-antigen binding system, with a subsequent intention to incorporate ionic liquids in a photomodulated antibody-antigen system. This is aimed at increasing versatility within the biotechnology described in this thesis. In general, the use of ionic liquids with very low vapour pressure will be beneficial to diverse applications in bioanalytical systems, in situations where current use in gaseous media including ultra-low pressures is not possible or is technically challenging.

5.3 Ionic Liquids: Deep Eutectic Solvents

The ionic liquids used within this study are further classified as Deep Eutectic Solvents (DES). As briefly mentioned in Chapter 1, Section 1.6, DES are types of ionic liquids whose mixture components result in melting point much lower than either of the individual components, rendering them effectively simple eutectics. DES can be formed between a variety of quaternary ammonium salts and carboxylic acids. The deep eutectic phenomenon was first described by Abbott *et al*., (2001; 2003) which involved 1:2 molar mixture of choline chloride (2-hydroxyethyl-trimethylammonium chloride) and urea. Choline chloride has a melting point of 302 °C and that of urea is 133 °C; the eutectic mixture however melts as low as 12 °C. The 3 types of ionic liquids (DES) used are: Glyceline 200 (a mixtures of glycerol and choline chloride [2:1 molar ratio]), Ethanline 200 (a mixture of ethane-diol and choline chloride [2:1 molar ratio]), and Maline 100 (a mixture of malonic acid and choline chloride [1:1 molar ratio]). These liquids were formed by heating the two components together at 100 ^{o}C until a homogeneous fluid formed (Abbott *et al*., 2003). Some of the physical properties of the 3 ionic liquids used are shown in Table 5.1. The term ionic liquids (instead of DES) will however be maintained throughout this thesis.

Table 5.1 : Ionic Liquids Involved in this Current Study.

Physical properties of the ionic liquids involved within study. Properties other than melting points were observed at 25 ^{o}C (Abbott *et al*., 2003).

Name	Composition	Density (g cm^{-3})	Viscosity (cP)	Melting Point (^{o}C)
Glyceline 200	Glycerol (66.66%) Choline chloride (33.33%)	1.20	259	-5
Ethanline 200	Ethane-diol (66.66%) Choline chloride (33.33%)	1.12	36	-20
Maline 100	Malonic acid (50%) Choline chloride (50%)	1.25	942	10

5.4 Chemicals, Biological Compounds, Materials and Equipment

All consumables (with the exception of the ionic liquids), biological compounds, materials and equipment were obtained as in previous chapters (Chapters 2, 3 and 4): Phosphate Buffered Saline (PBS) (Sigma Code: P4417-50tab), 3,3',5,5'– Tetramethylbenzidine (TMB) Tablets (Sigma Code: T 5525), Anti-Human Kappa Light Chains (Bound and Free)-Peroxidase Antibody Produced in Goat (Sigma Code: A7164), Atrazine (Sigma Code: 45330), Mouse Anti-FITC (Fluorescein) Monoclonal Antibody (Unconjugated) (Abcam code: ab2327), Albumin, Fluorescein Isothiocyanate Conjugate Bovine (FITC-Albumin) (Abcam code : ab47846), BSA-Atrazine recombinant scAb (University of Aberdeen/Haptogen), Donkey Anti-Mouse Secondary Antibody IGg-HRP (Cruz Marker Compatible Secondary Antibody) (Autogen Bioclear Code: Sc-2318), Immulon 4 ELISA plate, White LEDs, Based on GaN 27-33 Cd (Roithner Laser Technik Code: 5w4hca-H20-Ultra), UV LEDs (Roithner Laser Technik Code: NS360L-5RLO), Quartz Cuvette. The ELISA plate and UV-Vis spectra were read off Lab Systems iEMS Reader MF microtitre plate reader and UV-Vis Spectrophotometer UV-2100 (Shimadzu) respectively. The ionic liquids: Glyceline 200, Ethanline 200 and Maline 100 were obtained from ScioniX Ltd (University of Leicester).

5.5 Experimentation: Antibody-Antigen Binding in Ionic Liquids

5.5.1 Introduction

This study aimed to demonstrate for the first time the functioning of antibody-antigen binding in ionic liquids (Glyceline 200, Ethanline 200 and Maline 100). To illustrate this, the use of a standard microtitre plate based ELISA format, with the antigen immobilised to the solid phase and the antibody to bind in the liquid phase was employed. The ELISA format was slightly modified to allow only this key step (the reaction of the soluble antibody [binding to the immobilised antigen] in the liquid phase) to be performed with ionic liquids. The appropriate control of the water content, in order to maintain the antibody activity, of crucial importance, was observed (*i.e.* reactions were not observed in 100% ionic liquids).

Ionic liquids in general (including those involved in this study) are known to be viscous, hence an initial study to assess whether the viscosity of the ionic liquids employed had any influence on the rate of antibody binding in an ELISA. This was observed with a nominated antibody of study; Anti-Atrazine antibody fragment because of availability. Ionic liquid viscosity was not varied directly but via changes in the ratios of ionic liquid to an aqueous buffer (PBS). The ELISA procedure involved also varied the incubation period concerned with the antibody antigen binding step to observed effect on the rate of binding.

5.5.2 Methodology: ELISA

The binding ELISA involved within this chapter follows the same core steps of the ELISA described in Chapters 4; however some specific key steps are modified to aid ionic liquids inclusion analysis. The detailed protocol is as follow:

- Immulon 4 ELISA microtitre plate was coated with 100 µl/well of 10 µg/ml antigen in 1x PBS and incubated for 1 hour at 20 ˚C to immobilise antigen to the solid phase.
- The excess unbound antigen were removed from the plate's wells by a flip-slap emptying motion (*i.e.* holding the plate initially over a sink with the plate upside down in a rapid flipping motion, followed by a slapping motion of the plate upside down on a dry clean surface covered with absorbent paper (Kimwipe) to remove residual liquid content).
- The microtitre plate wells were blocked with 200 µl 1x PBS containing 1 % (w/v) skimmed milk (Marvel®) for 1 hour at 20˚C. (The removal of microtitre plate well content was via the flip-slap emptying motion)
- The plate was then washed twice with 200 µl 1x PBS containing 0.1 % Tween 20 (PBST) followed by a final wash with 1x PBS. (The removal of microtitre plate well content was via the flip-slap emptying motion)
- 100 µl of antibody at desired concentrations was added to the wells in triplicate and incubated for 1 hour at room temperature. (The removal of microtitre plate well content was via the flip-slap emptying motion) */**

- The plate was washed twice with 200 µl 1x PBS containing 0.1 % Tween 20 (PBST) followed by a final wash with 1x PBS. (The removal of microtitre plate well content was via the flip-slap emptying motion)
- 100 µl of an HRP labelled secondary antibody (to the primary antibody initially incubated) was added to the wells and incubated for 1 hour at room temperature. (The removal of microtitre plate well content was via the flip-slap emptying motion)
- The plate was washed twice with 200 µl 1x PBS containing 0.1 % Tween 20 (PBST) followed by a final wash with 1x PBS. (The removal of microtitre plate well content was via the flip-slap emptying motion)
- 1 3,3',5,5'-tetramethylbenzidine (TMB) tablet was dissolved in 10 ml of 0.05 M Citric Phosphate per Borate buffer of which 100 µl was added per well and allowed to develop (within 2 minutes) a blue colouration at room temperature.
- The reaction was quenched with 50 µl/well of 1M H_2SO_4 and read at 450 nm with Lab Systems iEMS Reader MF microtitre plate reader.

**This step was modified to allow the antibody to be presented into the microtitre plate wells at various ratios of ionic liquids to an aqueous buffer (PBS).*
*** The incubation period was varied with regards to the rate of binding analysis.*

Competitive Inhibition ELISA detailed protocol is as follow:

- Immulon 4 ELISA microtitre plate was coated with 100 µl/well of 10 µg/ml antigen in 1x PBS and incubated for 1 hour at 20 ˚C to immobilise antigen to the solid phase. (The removal of microtitre plate well content was via the flip-slap emptying motion)
- The microtitre plate wells were blocked with 200 µl 1x PBS containing 1 % (w/v) skimmed milk (Marvel®) for 1 hour at 20˚C. (The removal of microtitre plate well content was via the flip-slap emptying motion)
- Another set of wells of the microtitre plate were blocked with 200 µl 1x PBS containing 1 % (w/v) for 1 hour at 20˚C. (The removal of microtitre plate well content was via the flip-slap emptying motion)

- 100 µl/well of varying concentration of antigen were placed in the solely blocked wells in triplicate.
- 100 µl of antibody at a known fixed concentration was added to the wells containing the antigen and allowed to react for 12 hours at room temperature.*
- The reaction was transferred to the initial antigen coated well and allowed to react for 1 hour. (The removal of microtitre plate well content was via the flip-slap emptying motion)
- The plate was then washed twice with 200 µl 1x PBS containing 0.1 % Tween 20 (PBST) followed by a final wash with 1x PBS. (The removal of microtitre plate well content was via the flip-slap emptying motion)
- 100 µl of an HRP labelled secondary antibody (to the primary antibody initially incubated) was added to the wells and incubated for 1 hour at room temperature. (The removal of microtitre plate well content was via the flip-slap emptying motion)
- The plate was washed twice with 200 µl 1x PBS containing 0.1 % Tween 20 (PBST) followed by a final wash with 1x PBS. (The removal of microtitre plate well content was via the flip-slap emptying motion)
- 1 3,3',5,5'-tetramethylbenzidine (TMB) tablet was dissolved in 10 ml of 0.05 M Citric Phosphate per Borate buffer of which 100 µl was added per well and allowed to develop (within 2 minutes) a blue colouration at room temperature.
- The reaction was quenched with 50 µl/well of 1M H_2SO_4 and read at 450 nm with Lab Systems iEMS Reader MF microtitre plate reader.

**This step was modified to allow the antibody to be presented into the microtitre plate wells at various ratios of ionic liquids to an aqueous buffer (PBS).*

5.5.3 Ionic Liquids Effect on the Rate of Binding in an Antibody-Antigen Binding Reaction

One of the central assumptions to the analysis of antibody binding data is that the data is gathered after equilibrium is reached between the receptor (antibody) and ligand (antigen). This may take anything from minutes or several hours. Hence before a meaningful binding experiment can be done, the incubation time necessary to reach equilibrium was determined. Due to the high viscosity of ionic liquids, it is apparent that a standard ELISA protocol with the primary antibody incubation step of 1 hour at room temperature, may not allow the binding reaction to come to equilibrium. Although the ionic liquids employed in this study had varying viscosities, their viscosity effect were observed at ratios of 5 %, 50 % and 100 % of PBS content in the ionic liquid/PBS mix. This choice was to allow a control (100%), assessment of a mixed ionic liquid / aqueous solution (50%) and assessment of a near pure ionic liquid (5%) with the small water content to ensure constant solution properties during experimentation due to the hygroscopic nature of the ionic liquids chosen. The incubation (binding reaction) time for most assays is the time required for 90 % of the ligand to bind (Tramontano *et al.,* 1986; Ochola *et al.*, 2002). Within this study, the incubation time was varied by incubating a low concentration of the ligand (well below the presumed affinity constant [K_d]) with the receptor and observing the amount of ligand bound over time.

The experiment performed involved a modified ELISA (as described in Section 5.5.2) with varied antibody-antigen binding incubation period (over 24 hours), at a single antibody concentration of Anti-Atrazine antibody fragment (2.5×10^{-6} M), in the presence of 3 ionic liquids available (with different ratios of ionic liquid present in PBS [50 % and 95 % and 0 %/control]) to Atrazine–BSA conjugate (antigen). The resultant observation was noted in figure 5.1.

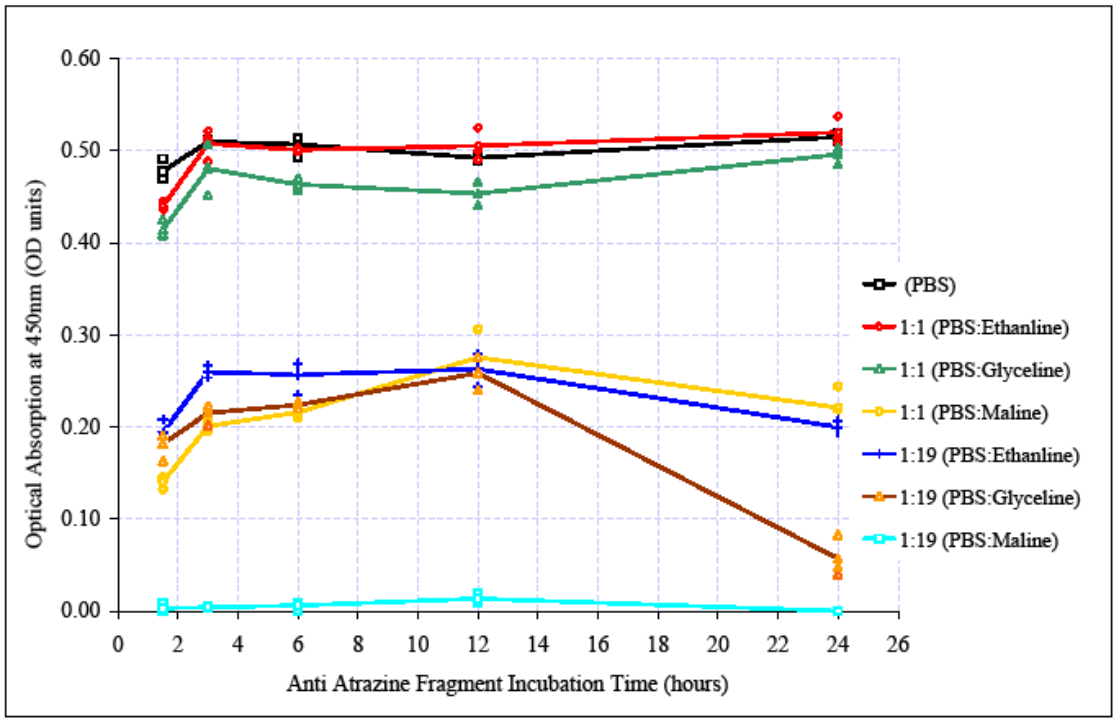


Figure 5.1 : Ionic Liquids Effect on the Rate of Antibody-Antigen Binding

Ionic liquids effect on the antibody-antigen rate of binding in a modified ELISA to assess binding properties, denoted by optical density (OD) readings at the end of reaction. The analysis also included various ratios of ionic liquid to an aqueous buffer (PBS).

Figure 5.1 shows that at 100 % aqueous buffer (control) and 50 % (ionic liquid in aqueous buffer) of Glyceline 200 and Ethanline 200, equilibrium is reached within 3 hours. 50 % (ionic liquid in aqueous buffer) of Maline 100 reached equilibrium at 12 hours but with 50 % less optical density (OD) signal compared to the control. 95 % (ionic liquid in aqueous buffer) of Ethanline 200 reached equilibrium at 3 hours but also with 50 % less OD signal compared to the control, however, 95 % (ionic liquid in aqueous buffer) of Glyceline 200 reached equilibrium at 12 hours with 50 % less OD signal but then declines in OD signal after 12 hours of incubation. 95 % (ionic liquid in aqueous buffer) of Maline 100 showed very little change in OD signal after 12 hours but then declines in OD signal after 12 hours of incubation. From these initial results it can be suggested that (although some reactions reached equilibrium within 3 hours), most solvent mixtures reached equilibrium with maximum ODs by 12 hours. This supports the necessity of the adjustment of the antibody binding step within the modified ELISA to 12 hours for the successive application within this thesis. With

regards to OD readings, it may be initially suggested that OD signal is inversely proportional to viscosity, however due to the observed decline in OD at some concentrations of ionic liquids; it suggests other factors within respective components of the employed ionic liquids may affect stability.

5.5.4 Antibody-Antigen Binding Interactions in Mixtures Containing Ionic Liquids

5.5.4.1 Introduction

This study deals with the binding nature of antibody-antigen in ionic liquids. It involves assessing the binding performance in ionic liquids with varying water content *i.e.* by 5%, 50% and 100%. To demonstrate to a limited extent the broad applicability of antibody-antigen in ionic liquids, two antibody-antigen systems were chosen (based mainly on their availability). A recombinant single chain antibody fragment (scAb) (Anti-Atrazine antibody fragment) which binds to Atrazine, and a monoclonal IgG (Anti-Fluoresceine Isothiocyanate [FITC]) which binds to Flureseine Isothiocyanate, were chosen to demonstrate the applicability of differing antibodies formats. Taking into consideration the ionic liquids effect on the rate of binding (Section 5.5.3), the antigen-antibody binding profiles in the ionic liquids were observed in a modified ELISA whereby the antibody-antigen binding incubation step was adjusted to 12 hours.

5.5.4.2 Anti-Atrazine Antibody Fragment Binding in Ionic Liquids

Anti-Atrazine antibody fragment binding profile via the modified binding ELISA procedure described in Section 5.5.2 was initially observed with all 3 ionic liquids (with different ratios of ionic liquid present in PBS [50 % and 95 % and 0 %/control]) to Atrazine–BSA conjugate (antigen). Eight antibody serial dilutions were prepared in the desired concentration range to assess binding profile, in this instance it was a 2 fold serial dilution to yield a K_d of ~ 0.6 x 10^{-4} M (~ 1.0 x 10^{-6} g/l)). A secondary HRP labelled antibody, human C-kappa light chain antibody (diluted by a factor of 1/1000 in 1x PBS) was used to detect the antibody-antigen complex formed. A $\log_{10}$ curve

plot of the mass concentration of antibody against optical absorption at 450 nm was observed to show the binding profile (Figure 5.2).

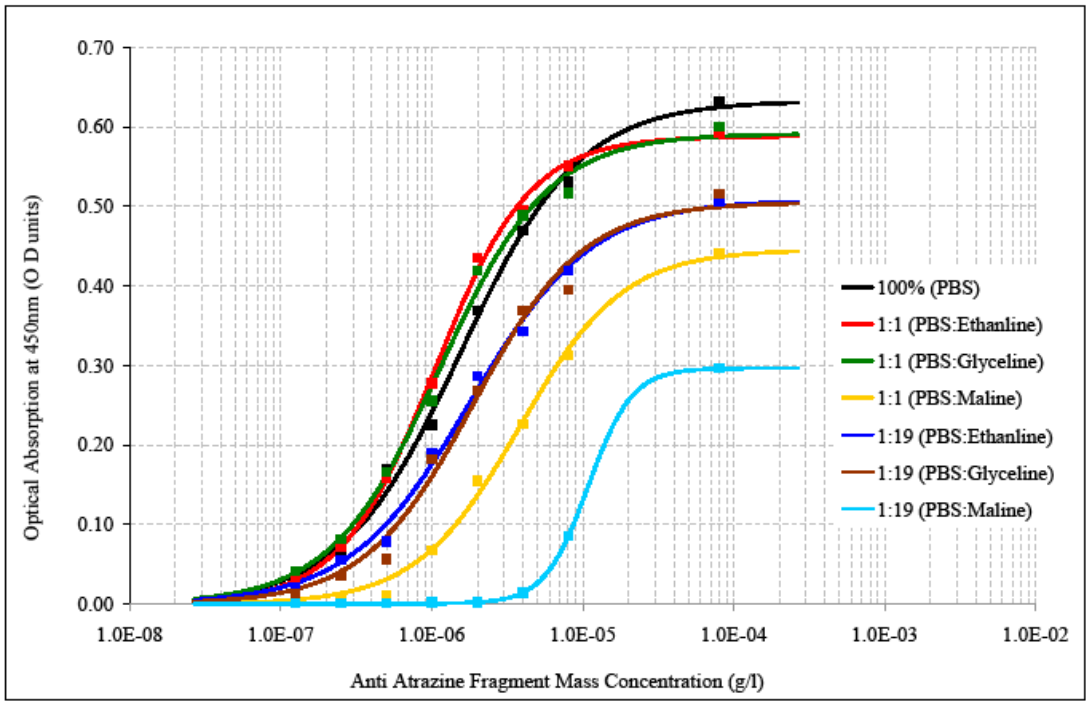


Figure 5.2 : Anti-Atrazine Antibody Binding in Ionic Liquids.

Comparison of IgG Anti-Atrazine fragment in an ELISA at different ratios of ionic liquids. 12 hours incubation period was applied within this assay.

Figure 5.2 displays a Microsoft Excel Solver Add-In feature used to determine the best-fitting sigmoid curve for the binding assay data. A sigmoid function was selected because this is the typical shape of a binding antibody assay curve. This allowed the antibody dissociation constant (K_d) (which is inversely proportional to the antibody binding affinity, K_a) to be measured. The K_d values for the antibodies are stated in Table 5.2. The typical range of K_d values for antibody-antigen binding between is 10^{-4} to 10^{-9} M (10^{-6} to 10^{-11} g/l) (Garcia *et al.*, 1999).

Table 5.2 : K_d values for Anti Atrazine in Ionic Liquids.

Solvent composition	K_d (g/l)
1:1 (PBS:Ethanline)	1.1×10^{-6}
1:1 (PBS:Glyceline)	1.1×10^{-6}
1:1 (PBS:Maline)	4.1×10^{-6}
1:19 (PBS:Ethanline)	1.8×10^{-6}
1:19 (PBS:Glyceline)	1.9×10^{-6}
1:19 (PBS:Maline)	1.1×10^{-5}
100% (PBS) (Control)	1.0×10^{-6}

From the binding profile in Figure 5.2 and Table 5.2, there was no significant difference in K_d of the control (100 % PBS buffer) and 50 % (ionic liquid in aqueous buffer) of Glyceline 200 and Ethanline 200, however 50 % (ionic liquid in aqueous buffer) of Maline 100 show a 4 fold difference in the K_d compared to the control, also the OD signal readings was lower than that of the control. 95 % (ionic liquid in aqueous buffer) of Glyceline 200 and Ethanline 200 showed similar results with an equilibrium binding constant 2 folds lower than that of the control and also with a lower OD signal (but not as low as the 50 % Maline 100 in aqueous buffer). 95 % (Maline 100 in aqueous buffer) displayed a 10 fold difference in equilibrium binding constant and the lowest OD signal (50 % lower than the control). A detailed explanation for these observations is not apparent at present but these results show that high affinity antibody binding is possible in ionic liquids and that the detailed nature of the ionic liquid can affect its function.

Due to nature of Maline 100 on the antibody binding properties, its effect on the rate binding (section 5.5.3), and the low OD signal it displayed (which may be as a result of destruction of biomolecules by possible means such as denaturing of protein, viscosity effect or ionic stripping of the microtitre plate wall) it was decided upon to leave out further analysis involving Maline 100 within this thesis. The scope of study

needs to be widened to deliver further investigations, but due to limited resources and time available, this was not conducted.

Following the binding profile of Anti-Atrazine in ionic liquids, the next logical step was to observe a competitive inhibition ELISA. This was conducted (as described in Section 5.5.2) with an anti-Atrazine concentration of 2.5 x 10^{-4} M with free Atrazine (antigen) concentrations ranging between 1.4 x 10^{-4} M and 1.4 x 10^{-15} M. Due to the observed Maline 100 effect on the binding of antibody-antigen, only Glyceline 200 and Ethanline 200 ionic liquids (containing 5%, 50% and 100% of aqueous buffer) were involved in this study. A $\log_{10}$ plot of the molar concentration of antigen versus optical absorption at 450 nm was observed (Figure 5.3).

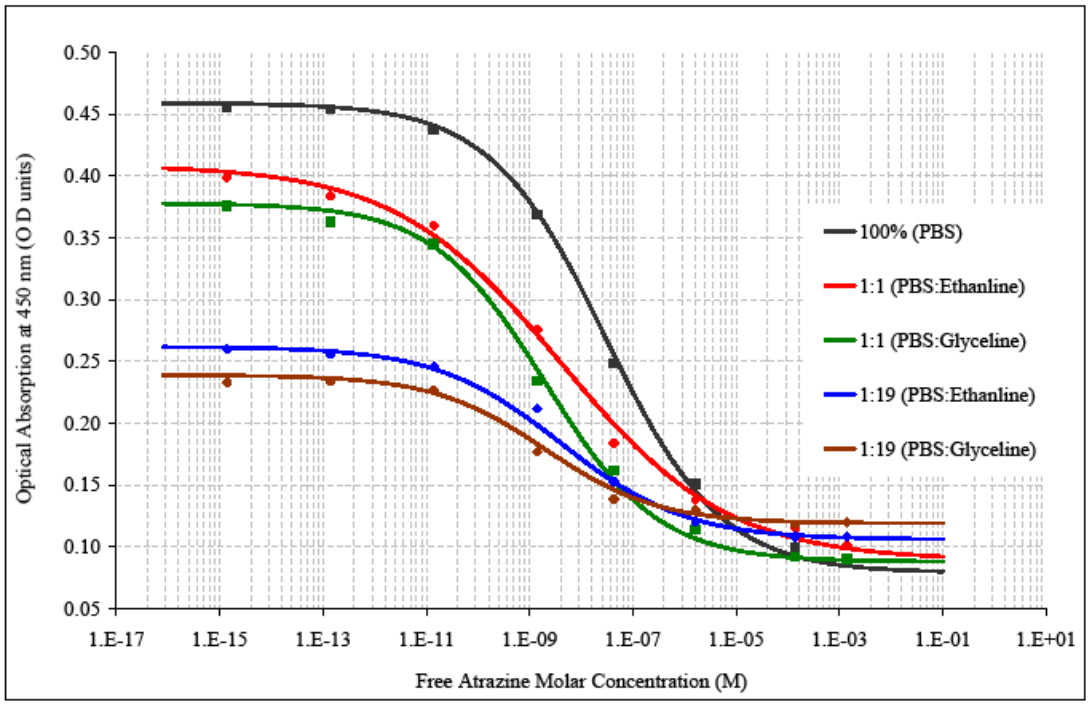


Figure 5.3 : Molecular Specificity of Anti Atrazine in Ionic Liquids.

Competitive inhibition ELISA of Anti-Atrazine (2.5 x 10^{-4} M) in ionic liquids containing 5%, 50% and 100% of aqueous buffer.

The Microsoft Excel Solver Add-In feature was used to determine the best-fitting sigmoid curve for this data. The inhibitory concentrations at 50% binding (IC_{50}) values (as explained in Chapter 4) for the antibodies are stated Table 5.3.

Table 5.3 : IC_{50} values for Anti Atrazine in Ionic Liquids.

Inhibitory concentrations at 50% binding (IC_{50}) were obtained from Figure 5.3

Solvent composition	IC_{50} (M)
1:1 (PBS:Ethanline)	8.0×10^{-8}
1:1 (PBS:Glyceline)	7.1×10^{-8}
1:19 (PBS:Ethanline)	1.8×10^{-9}
1:19 (PBS:Glyceline)	2.9×10^{-9}
100% (PBS) (Control)	3.0×10^{-8}

Figure 5.3 and Table 5.3 shows that the binding profile of ionic liquids of Glyceline 200 and Ethanline 200. At 50 % (ionic liquid in aqueous buffer) there is ~ 2 ½ fold difference in IC_{50} value compared to the control (100 % PBS buffer). 95 % (ionic liquid in aqueous buffer) of Glyceline 200 and Ethanline 200 showed 10 fold difference in the IC_{50} value compared to the control; however the OD signal was much lower. The most common problem to deal with in receptor-ligand interactions is non-specific binding. This is related to the presence of low affinity binding sites and also to binding to a set of identical and independent sites that have an affinity and capacity for the ligand in question. The observations within these binding studies indicate that there was no significant evidence of non-specific binding via the competive ELISA.

5.5.4.3 Anti-FITC Binding in Ionic Liquids

To demonstrate the applicability of differing antibodies formats, Anti-FITC was used in place of Anti-Atrazine and the binding ELISA profile was observed. This study involved Glyceline 200 and Ethanline 200 (containing 5%, 50% and 100% of aqueous buffer). The binding ELISA assay included FITC-Albumin as the antigen. The antibody (Anti-FITC) was serial diluted to yield a K_d of ~ 0.6×10^{-6} M (~1.0×10^{-8} g/l). A secondary HRP labelled antibody, Anti-Mouse IgG (diluted by a factor of 1/1000 in 1x PBS) was used to detect the antibody-antigen complex formed. A $\log_{10}$

plot of the molar concentration of antibody versus optical absorption at 450 nm was observed to show the binding profile (Figure 5.4).

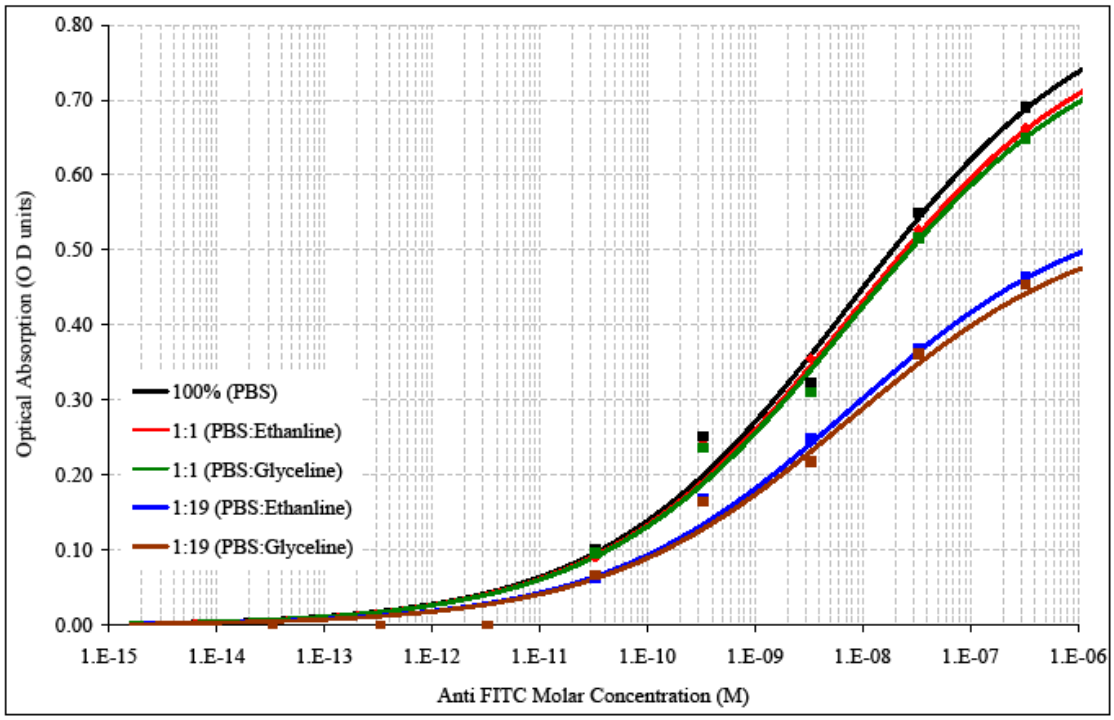


Figure 5.4 : Anti-FITC in Ionic Liquids.

Comparison of IgG Anti-Fluoresceine Isothiocynate (FITC) in an ELISA at different ratios of ionic liquids. 12 hours incubation period was applied within this assay.

Figure 5.4 displays a Microsoft Excel Solver Add-In feature used to determine the best-fitting sigmoid curve for the binding assay data. A sigmoid function was selected which allowed the experimental K_d to be determined. These K_d values for the antibodies are stated Table 5.2.

Table 5.4 : K_d values for Anti-FITC in Ionic Liquids.

Solvent composition	K_d (g/l)
1:1 (PBS:Ethanline)	3.1×10^{-8}
1:1 (PBS:Glyceline)	3.2×10^{-8}
1:19 (PBS:Ethanline)	6.0×10^{-8}
1:19 (PBS:Glyceline)	6.1×10^{-8}
100% (PBS) (Control)	3.0×10^{-8}

Figure 5.4 and Table 5.4 shows the binding curve with corresponding K_d values of the IgG Anti-Fluorescein Isothiocynate (Anti-FITC) in a binding ELISA with ionic liquids: Glyceline 200 and Ethanline 200 (containing 5%, 50% and 100% of aqueous buffer). At 50 % (ionic liquid in aqueous buffer) of both Glyceline 200 and Ethanline 200, there was no significant difference in the K_d value compared to the control (100 % PBS buffer). 95 % (ionic liquid in aqueous buffer) of both Glyceline 200 and Ethanline 200 slowed 2 fold difference in the K_d value compared to the control; however the OD signal was ~ 35% lower. These initial results obtained indicate that the ionic liquid including an alternative antibody (Anti-FITC) study showed similar results to the previously studied Anti-Atrazine antibody fragment (Section 5.5.4.2).

The work presented in this thesis however does not show a competitive inhibition ELISA for Anti-FITC due to limited available resources and therefore needs to be considered for future work. In general, the initial experimentation of antibody-antigen binding in Glyceline 200 and Ethanline 200 to content of 50 % and below in aqueous buffer, produced identical results to that of the standard aqueous PBS (control). At 95 % ionic liquid content, a lower level of binding activity was observed. The similarity of results between the antibody types implies that the observed phenomenon is reproducible and may be generic. Further test on other antibodies will certainly need to be performed to verify this hypothesis.

5.5.5 Spiropyran Dyes in Ionic Liquids

5.5.5.1 Introduction

The overall intention within this thesis is to apply ionic liquids as solvents types involved in the photomodulation of the antibodies. To achieve this, the antibody needs be able to function in ionic liquids and just as importantly the photochromic dyes needs to also function in ionic liquids. This study aims to observe the photoswitching properties of the synthesised spiropyran dye (SP-COOH) (from Chapter 2). Since Glyceline 200 and Ethanline 200 were the two most favourable ionic liquids that supported antibody-antigen binding, photoswitching of SP-COOH was observed in these only.

5.5.5.2 Spiropyran Dyes Photoswitching in Ionic Liquids

Spiropyran dyes are soluble in most organic solvents but do not dissolve very well in aqueous solutions and, as initial observation shows, also in the ionic liquids employed in this study. Therefore this study involved dissolving SP-COOH in ethanol to aid solubility and made up with the desired ionic liquid to observe photoswitching. 0.25 μg/ml of SP-COOH in 1:9 ionic liquid to ethanol was observed for Glyceline 200 and Ethanline 200. Photoswitching of the dye was observed as described in Chapter 2 Section 2.5.2, *i.e.* the solution was initially exposed to the white LEDs to trigger the conversion to the spiropyran form for 3 minutes. 1.5 ml of this solution was placed in a quartz cuvette and with appropriate solvents as blanks, the dye solution was observed between 400 and 700 nm using a UV-Vis Spectrophotometer. The samples were then placed in a dark environment at 20 °C for 12 hours to allow dark adaptation to the merocyanine form of the dye and observed again between400 and 700 nm. These were observed in triplicates.

The initial spectrum for photoswitching of SP-COOH in Glyceline 200 and Ethanline 200 (with 90 % ethanol) is shown in Figure 5.5.

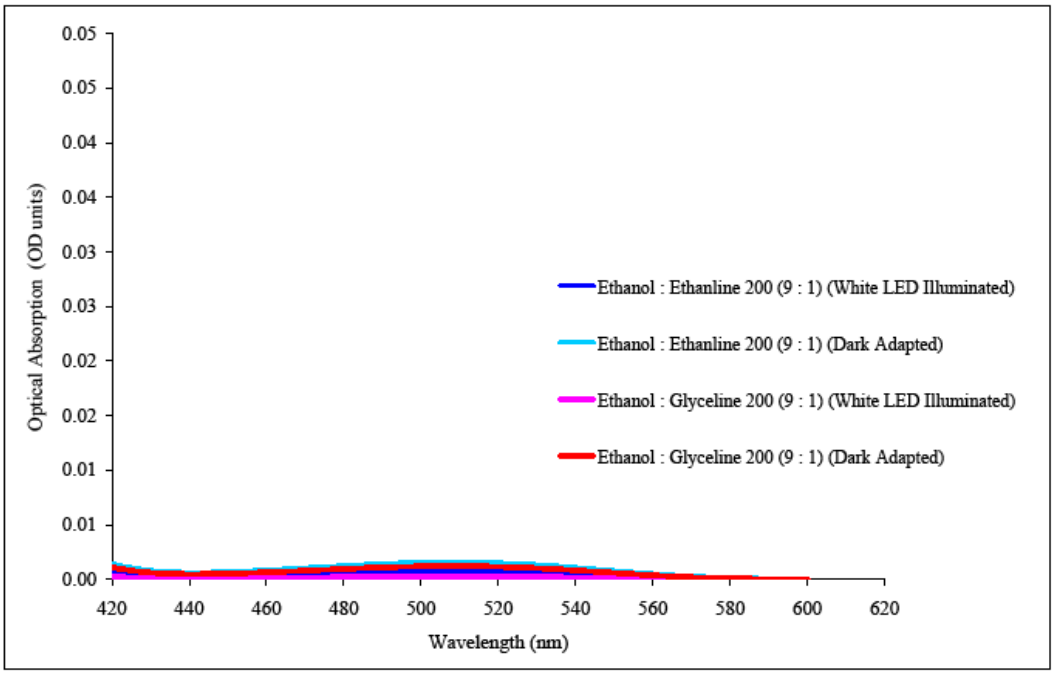


Figure 5.5 : Spiropyran Dyes Photoswitching in Ionic Liquids.

0.25 µg/ml of spiropyran dye in ethanol:ionic liquid (Glyceline 200 and Ethanline 200) at (9:1) at 20 °C. The solutions were dark adapted and white light illuminated to allow merocyanine and spiropyran conversion respectively, and observed between 400 and 600 nm.

The results attained in Figure 5.5 indicated that the dye failed to switch forms after illumination and remained in the colourless spiropyran form even at a high concentration of ethanol (90 % ethanol to 10 % ionic liquid). Repeat analysis showed the same results. It was then hypothesised that the ionic properties of the Glyceline 200 and Ethanline 200 prevents the spiropyran dyes from switching forms, and was concluded that on this occasion, inclusion of ionic liquids to a photomodulation analysis of a modified protein will not be possible.

5.6 Discussion

5.6.1 Antibody-Antigen Binding in Ionic Liquids

The proposal to utilise ionic liquids (Glyceline 200 and Ethanline 200) as solvents in antibody-antigen reaction was confirmed to be feasible. The results obtained for the ELISA analysis of the antibody-antigen interaction in the presence and absence of the ionic liquids indicated, fundamentally, the antibody dissociation constant (K_d) of the standard profile of the antibodies of study were not any different with up to 50 % ionic liquid present during binding reaction. Also at higher an ionic liquid concentration (*e.g.* of 95 % ionic liquids in solvent mix), antibody-antigen binding is still feasible but at an decreased optical density (OD) signal - this decrease could imply that the level of water present may regard the environment as near-extreme conditions (hence affect the biological activity); the ionic nature of the solution may contribute to surface stripping of the immobilised complex formed on the well of the microtitre plate; or even so the ionic nature may interfere with the binding capability of the antibody and/or antigen.

With regards to the time taken for the antibody-antigen binding reaction in the presence of ionic liquids to reach equilibrium, it be can suggested that there is a link between the concentration of the ionic liquids to the time taken for reaction to reach equilibrium; however it was also observed that the length of time which the antibody-antigen binding reactions are exposed to ionic liquids may trigger secondary negative effects as observed in Section 5.5.3 – the decline in activity may be because of the near-extreme condition / ionic stripping described. This could imply that the ions in the solutions maybe start to have an effect on the stability of the antibody over time.

Ionic liquids can be fine-tuned to specificity Abbott *et al*., (2001), however some ionic liquids *e.g.* Maline 100 (of study) may show the other extreme; imcompatible with certain applications. Maline 100 may be a good candidate for other applications but not for this study. Nevertheless, it has been successfully demonstrated that antibody-antigen binding is feasible in some examples of ionic liquids (Glyceline 200 and Ethanline 200). The idea of antibody-antigen technology in applications to detect

volatiles, in gas phase sensing, in operation in icy environments, fluid handling and assay in low pressures may be feasible with the use of ionic liquids as solvents. In summary, this study includes work that has advanced the current application of ionic liquids in biomolecules.

5.6.2 SP-COOH in Ionic Liquids

Carboxylated spiropyran dyes synthesised failed to photoswitch in a solution (9:1) of ethanol in either Glyceline 200 or Ethanline 200. This mixture was predominantly an organic mix (of 90 % ethanol with 10 % ionic liquid) with very low concentration ionic liquids. The observed results indicate that the spiropyran dye may be locked in one form *i.e.* the colourless spiropyran from in the smallest amount of ionic liquids. As stated in Chapter 1, merocyanines are zwitterionic by structure; hence the ionic nature of the net solution may be preventing the formation of the merocyanine form of the dye as a result of interactions between the ions. Lin, (2003) stated that formation of merocyanine depends greatly on the viscosity and the polarity of the solvent. In the context of viscosity, based on the percentage of ethanol in solution (90%) compared to ionic liquids, it can be suggested that viscosity may not necessarily have that critical a role in influencing photoswitching but rather the polarity (ions present).

The inability to observe photoswitching of SP-COOH in ionic liquids suggests further observing experimentation with other forms of ionic liquids, however due to insufficient experimental time and resources, this aspect will need to be considered as future work.

5.7 Conclusions

Antibody-antigen binding reactions for two antibody types (Anti-Atrazine and Anti-FITC) have been proven, for the first time, to be feasible in various concentrations of ionic liquids (Glyceline 200 and Ethanline 200). Further tests will need to be performed to expand these observations on other range of antibodies. The attempt to photoswitch spiropyrans dyes in the presence of ionic liquids was not feasible. The ionic nature of ionic liquids may have hindered the switching nature of the spiropyran

dye. Further tests will need to be performed to confirm these observation since a different range of ionic liquids may be better suited.

Chapter 6

FINAL DISCUSSIONS, CONCLUSIONS AND FUTURE WORK

6 Final Discussion, Conclusions and Future Work

6.1 Final Discussion

Since the four sections of practical work were carried out and reported as a series of experiments in which the results of each experiment and implications for subsequent work were discussed throughout Chapters 2 to 5, this final discussion presents a brief summary of the developments made and the relationship each area of work has on the conclusion of this PhD work.

6.1.1 Synthesis and Characterisation of SP-COOH

The synthesis of a carboxylated spiropyran dye was a very important step within this study. The synthesis of SP-COOH; consisting of a carboxyl terminated alkyl chain suitable for carbodiimide-mediated conjugation to free amine groups present on proteins was successful. The procedure employed yielded high product recovery. The SP-COOH-Me derivative failed to deliver what was expected, since SP-COOH-ME was expected to enhance the water solubility of the dye. The immediate characterisation of observing photochromism in ethanol showed rapid conversion rate between isomers. The various characterisation steps taken to authenticate the product synthesised was sufficient, however, ^{13}C NMR analysis characterisation step was not feasible on this occasion although it was anticipated that the unknown behaviour of the dye under the ^{13}C NMR analysis may be have contributed to this observed reponse as also stated within the findings by Bouas-Laurent *et al.*, (2001). The stimulus for triggering the synthesised spiropyran dye isomeric state had to be adjusted to accommodate the need for the dye in an aqueous environment – it was established that UV illumination mode of switching was ineffective when the spiropyran dyes were in aqueous buffers such as HEPES, PBS or MES. This resulted to an alternative mode of stimulus for triggering photochromism i.e. the slow coversion of dye to the merocyanine isomeric form in the dark and the rapid coversion of the dye to the spiropyran isomeric form in white light. It may be concluded that the dark adaption technique of merocyanine isomer conversion is significantly slow and as such may not be ideal for rapid switching application, which is a desired function of dye

photoswitching. Nonetheless is has been established that the synthesised dye can undergo photochromism in 90% aqueous buffers of HEPES, PBS or MES. The behaviour of solvent properties also highlights the need care of application of SP-COOH. Fatigue assessment revealed that stability SP-COOH was not affected significantly.

6.1.2 Photomodulation of HRP Activity

Photomodulation of HRP (which served as a trial run to formulate conditions which would favour antibody activity photomodulation) had been previously observed by Weston et al., (1999) whereby 92 % modulation was observed. Sesay, 2003 also conducted this investigation and yielded 72 % modulation. Both their mode of switching was via visible light and UV illumination. In contrast, this current study gave modulation of up to 60 % and illumination was via white LEDs and dark adaptation as a result of UV light, which was not producible under the desired aqueous conditions (10% ethanol, 90% aqueous buffer).

Carboxylated spiropyran are known to exhibit negative photochromism and this was advantageous in ensuring that switching of the two isomers was feasible despite not being rapid or activated via UV as desired. The solvent effect on spiropyrans is known to vary and is a very important study in its own right. Consequently the behaviour of the spiropyran dye in solvent, in favour of its application, needs to recognised within each application as evident. Despite the modulation of HRP not being as concretely proven as previous work undertaken, the experimental conditions were different, hence a possible explanation for the discrepancies and the observed findings. It is believed that the change in configuration of the dye has disrupted the structure of the protein, thus possibly inducing a subsequent change in shape of the binding or active site of the protein which gave the observed findings.

Muranaka *et al.*, (2002) suggested that photomodulation of enzymes such as HRP is not based on the environmental change of the polarisation by the cis/trans photoisomerisation of the dye. Muranaka *et al.* suggested it was based on a side chain

conformational change that affects the substrate binding site or the entire protein conformation. However with the results attained, bearing in mind that the number of dyes attached to the protein did not necessarily influence modulation, it is proposed that the environmental changes might have been influential. The ambiguity in considering deducing the exact mechanism of modulation therefore remains; this might however be due to the varying mechanism with respect to target proteins.

6.1.3 Photomodulation of Antibody Affinity

The main aim of this study, which was to synthesise a photochromic antibody whose affinity could be modulated reversibly by photons, has been established. The use of spiropyran dye modified HRP to scale up the protocols involved has served its purpose. It is reported here with 5 different antibodies whose affinity to their respective antigens can be modulated by the aid of photochromism of attached spiropyran dyes. Although the degree of modulation varied, the principle remained. Lui *et al*., (1997) stated that within most studies of photomodulation of proteins, chemical modification of the protein is employed via non-specific target groups (*e.g.* lysine groups) that couple many photochromic units (consisting of a carboxyl terminated alkyl chain) to undefined sites on the protein (just as described within this thesis). Lui *et al*. suggested that a more rational design of photomodulated protein would be to have photochromic dye groups incorporated site-specifically, *i.e.* at key sites on a protein structure. This will henceforth give more insight on how photomodulation is achieved and also aid enhancement of the application of photochromic dyes in protein function modulation.

The use of covalently attached photochromic spiropyran dyes to proteins (HRP and antibodies) has proven its ability to influence a significant degree of activity modulation when appropriately illuminated. The antibodies modified generally retained their activity after conjugation with the dye implying the method of coupling was not overly invasive. These intial results suggest a significant step to towards an immunoaffinity assay system implementing photosensitive antibodies. Future application in the capacity of regenerating bio-sensing surfaces via these modified

antibodies may also be practicable although a reversible photomodulated study may need to be completed.

6.1.4 Photodegradation (Fatigue) of SP-COOH

Fatigue assessment of the photoswiching profile of unconjugated dyes (SP-COOH) shows low levels of fatigue in comparison to the conjugated dye to proteins (SP-HRP and SP-Ab). Conjugated spiropyran dye fatigue was evident throughout all modified protiens. The immediate switching of the conjugated spiropyran dye from the SP to MC form remained stable during the first couple of photoisomerisation analyses, however this was followed by a noticeable gradual photodegradation of the photochromic nature of the dye in subsequent photoswitching. The conjugated dye merocyanine switching was assessed over a 12 hours period to enable conversion which is different from an immediate switch with UV light source established by others published findings; *i.e.* a lag incubation period may contribute to fatigue as well. The stability testing (fatigue over 10 weeks period) although was much longer when compared to the immediate switching analysis showed a higher magnitude degradation over time. This could have been influenced by the freshness of the solvent (aqueous buffer) such that the possibly microorganism growth could have contributed to some form of interference with photoswitching. Overall these results attained wtihing this study suggests that fatigue with respect to SP-COOH conjugated to antibodies and HRP may be due to: *(i)* the conjugation reaction treatment, and/or *(ii*) the molecular instability of the spiropyran dye after it was coupled to the antibody *(iii)* and/or the nature of the solvent environment they are in.

6.1.5 Antibody-Antigen Binding in Ionic Liquids

The demonstration of antibody-antigen binding in various ratios of ionic solutions was confirmed feasible for the first time. A major advantage of ionic liquids; the ability to fine tune ionic liquids by altering the cation, anion and attached constituents to manipulate the solvents physical and chemical properties renders their applications as versitle - the application within this study is only based on a few types of ionic liquids. The properties and behavior of the ionic liquid can be adjusted to suit an

individual reaction type; they can truly be described as designer solvents - although not within the scope of this study. By choosing the correct ionic liquid, high product yields of chemical reaction can be obtained, and a reduced amount of waste can be produced in a given reaction. Often the ionic liquid can be recycled, and this leads to a reduction of the costs of the processes. Within this application, the feasibility of antibody-antigen binding in the available range of ionic solutions was assessed rather than the creation of designer solvents. Nonetheless the application of ionic liquids in this content has set the precedent with antibody-antigen binding in ionic liquids.

6.1.6 Spiropyran Dyes in Ionic Liquids

Despite the interesting results attained with regards to antibody-antigen binding in various ratios of ionic liquids to aqueous buffers, the same cannot be said about photochromism of spiropyrans. Spiropyrans are a class of photochromic molecules whose structure is zwitterionic (*i.e.* when in the merocyanine form), hence the presence ions in the solution can cause the molecule to behave otherwise, *i.e* interact with the ions of the ionic liquids. It is believed that the ionic nature of ionic liquids does not support photochromism of spiropyrans. Spiropyrans are known to behave differently in protic, aprotic, non polar and aqueous solvents; hence the behaviour in ionic solutions was anticipated to raise concerns as observed.

6.1.7 Summary

The used of HRP to set up protocols to aid asseses the feasibility of photomodulate antibody was successful; it helped provide means of opbserving photomodulation in acqeuous buffers. Antibody affinity control aimed for affinity biosensors seems feasible - despite not observing 100% modulation, ~ 65 % modulation implies that less concentration of harsh solvent (wash buffers) can be introduced to the modified antibodies during regeneration if applied in biosensors. Despite loss of rapid photoswitching due to the solvents employed, optimisation of technique will only benefit this observation. It can however be emphasized that photochromic antibodies are practicable. The feasibility of antigen-antibody in ionic liquids may not be

necessarily be benefitial with respect to photochromism, but this findings can pave the way to the manufacture of vacuum biosensor or biological assaying of in space.

6.2 Final Conclusion

6.2.1 Photomodulation of HRP Activity

Modification of HRP with spiropyran dyes in 90% aqueous buffer and 10 % ethanol resulted in a 60 % modulation of protein activity by merocyanine isomer of the dye. The activation of the isomers of the dyes was by visible light (with LEDs) and dark adaptation resulting to the spiropyran and merocyanine forms respectively. Visible light illumination of the dye-protien complex was respondsible for the decrease of enzyme activity and dark adapation was responsible for reverting or maintaining the enzyme activity at its native level.

6.2.2 Photomodulation of Antibody Affinity

The variety of antibodies modified by spiropyran dyes that can be photomodulated ranged from monoclonals, polyclonals and antibody fragments. The extent of modulations varied between antibodies, however the BSA conjugated whole IgG antibodies were photomodulated by the merocyanine isomer of the dye whiles the non BSA conjugated antibodies were modulated by the spiropyran isomer. These findings can provide new opportunities for controlling activity in biosensors, diagnostics, affinity separations, bio-processing, therapeutics, and bioelectronics applications.

6.2.3 Photodegradation (Fatigue) of SP-COOH

The fatigue effect is a common feature observed among a wide range of organic photochromic compounds, and was hypothesised to be caused by photodegradation of the dyes under UV irradiation. The finding within this study (which included the omission of UV illumination) did not improve circumstances as fatigue was still evident. This however suggestes that fatigue is influenced by *(i)* the conjugation reaction treatment, and/or *(ii)* the molecular instability of the spiropyran dye after it was coupled to the antibody.

6.2.4 Antibody-Antigen Binding in Ionic Liquids

The results obtained for the ELISA analysis of the antibody-antigen interaction in the presence of the ionic fluids indicated a considerably important point of application; *i.e.* the use of antigen-antibodies technology in low vapour pressure conditions such as space assaying. This finding can serve as a basis to build a more significant application of ionic liquids in conjunction with the antibodies.

6.2.5 Spiropyran Dyes in Ionic Liquids

Spiropyrans are not photochromic in ionic liquids. The dye molecules are locked in the spiropyran form when in ionic liquids with a concentration as low as 10 % (ionic liquids: ethanol, 1:9).

6.2.6 Summary

- Photomodulation of HRP has been re-confirmed to be feasible in predominatly acqeous solution.
- Photomodulation of 5 different antibodies has been feasible for the first time.
- Antigen-antibody reaction in various ratios of ionic solutions is feasible.
- Photochromism of spiropyrans dyes in the ionic liquids witihin this study is not feasible.

6.3 Future Work

6.3.1 Substitution of Spiropyran Dyes with Spirooxazines

Spirooxazines are very similar to spiropyrans in many ways. The only structural difference is that the C=C in pyran is replaced by C=N in oxazine (Lin, 2003). However one interesting differential feature is fatigue. Spirooxazines have better fatigue resistance, as also stated by Baillet *et al.*, (1993), they have attracted considerable interest because of their good fatigue resistance under a long period of irradiation in comparison with spiropyran derivatives; hence this is something worth looking into in the long term application of photomodulation of antibodies.

6.3.2 Regeneration of Modified Protein after Photomodulation

This current study did not specifically observed photomodulation of protein function over more than 3 cycles *i.e.* "on" "off" and "on" again. For future application, such as biosensors technology, the renegeration of antibodies is of high significance. Hence it may be of importnace to study the reversibilty activity cycle of the photomodulated modified proteins. This may be achievable with antibodies applied in an SPR system.

6.3.3 Specific Protein Site Photomodulation & Incorporation of the SP-Ab in Immunoaffinity Biosensors

Despite success with photomodulation of the proteins via spiropyran dyes within this study, site specific target of dye attachment to protein can help define photomodulation rather than dependance on random coupling via lysine groups available (which can vary from protein to protein). Key-site photomodulation can also help precisely acknowledge the mechanism of action.

Affinity biosensors offer real time kinetics observation; this can be an ideal path to observe how photomodulation is achieved on modified antibodies. With regards to the the general feasibility of photomodulation of antibodies in a biosensor, an applied version of photochromic antibodies in a Surface Plasmon Resonance (SPR)

technology as in an immunoaffinity biosensor would be a step closer to the establishment for a reagentless biosensor.

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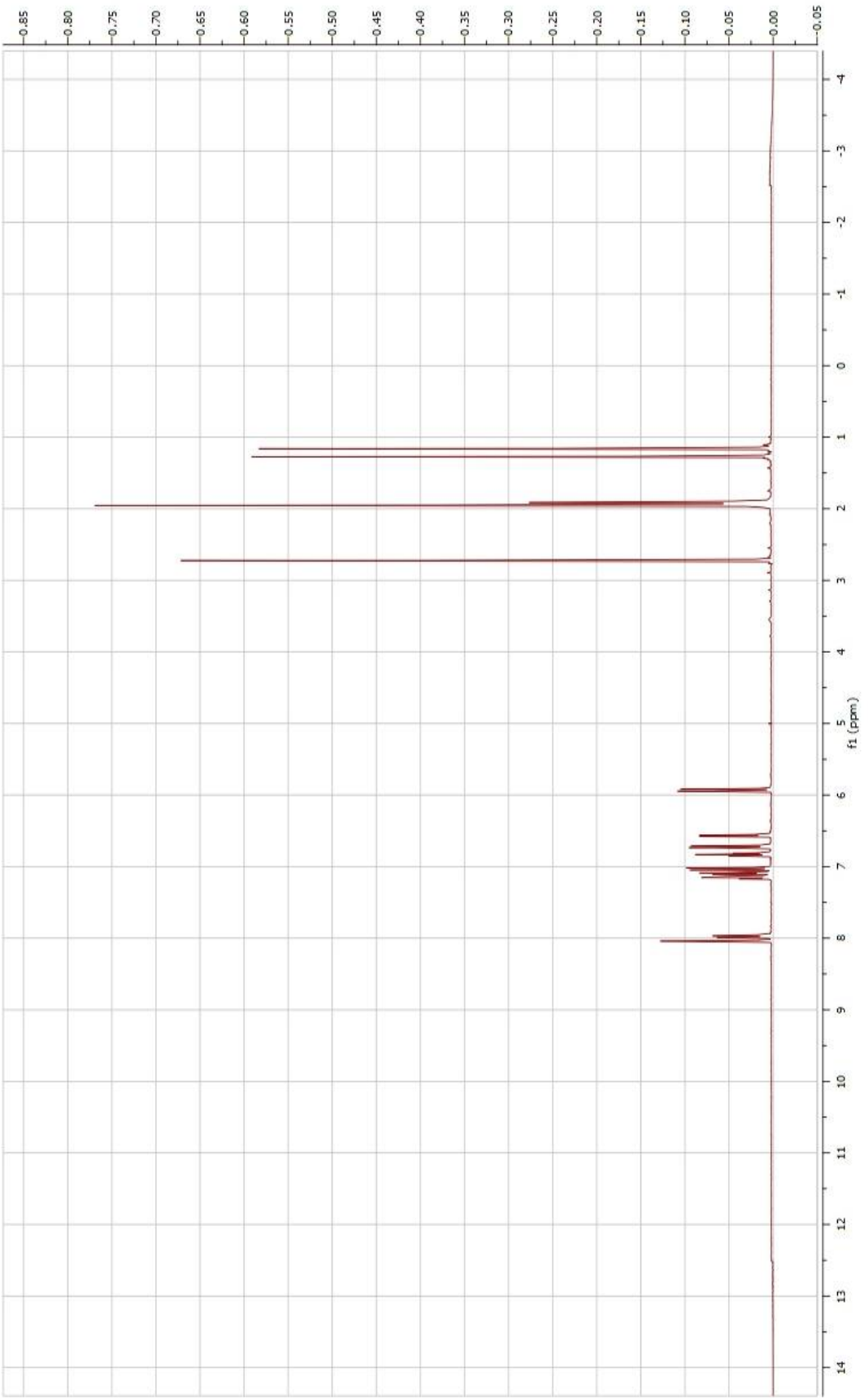
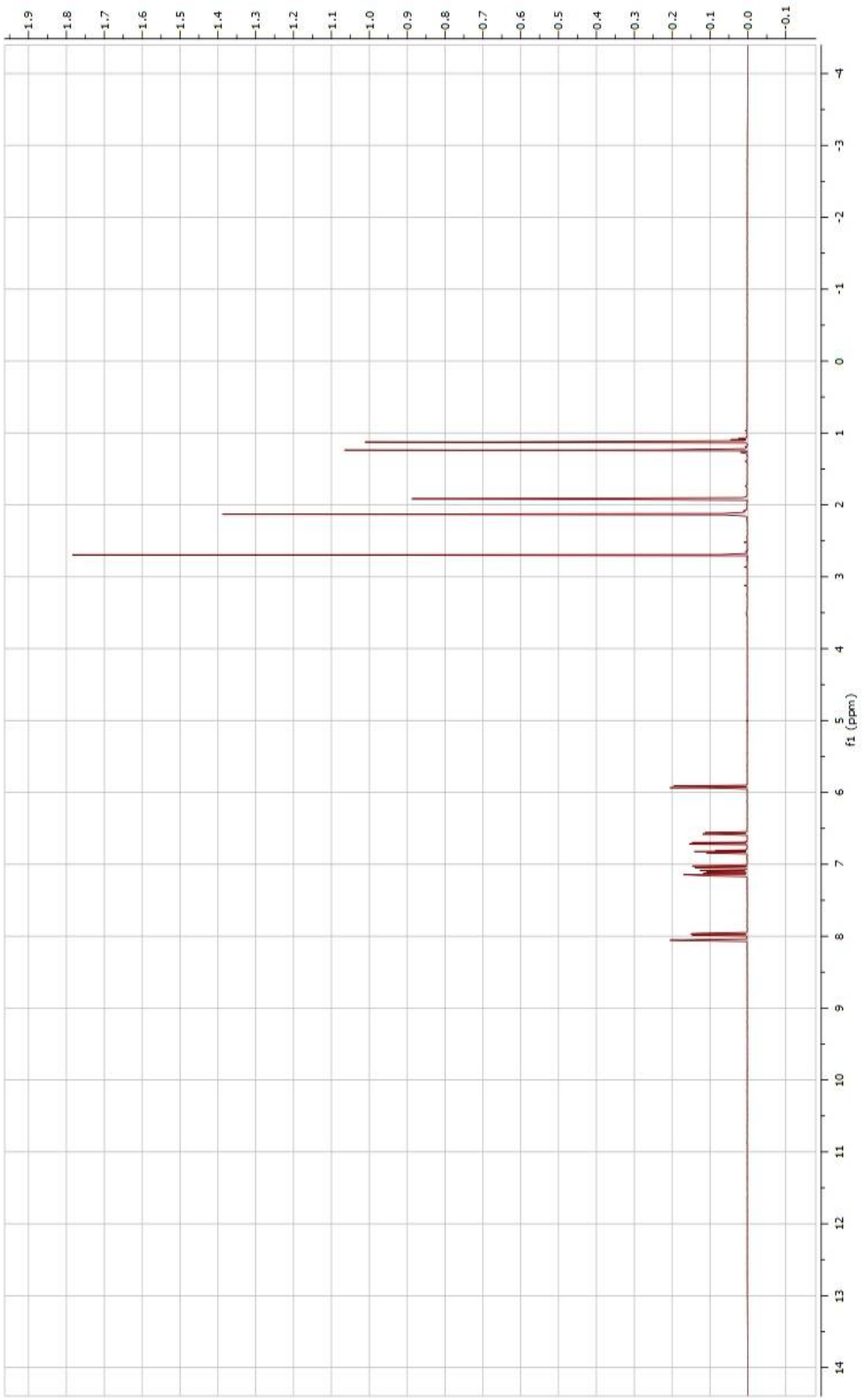
Appendix

NMR Spectra:

NMR (SP-Sigma) ^{1}H NMR (400 MHz, ACETONITRILE-D3)



NMR (SP-COOH) ^{1}H NMR (400 MHz, ACETONITRILE -D3)



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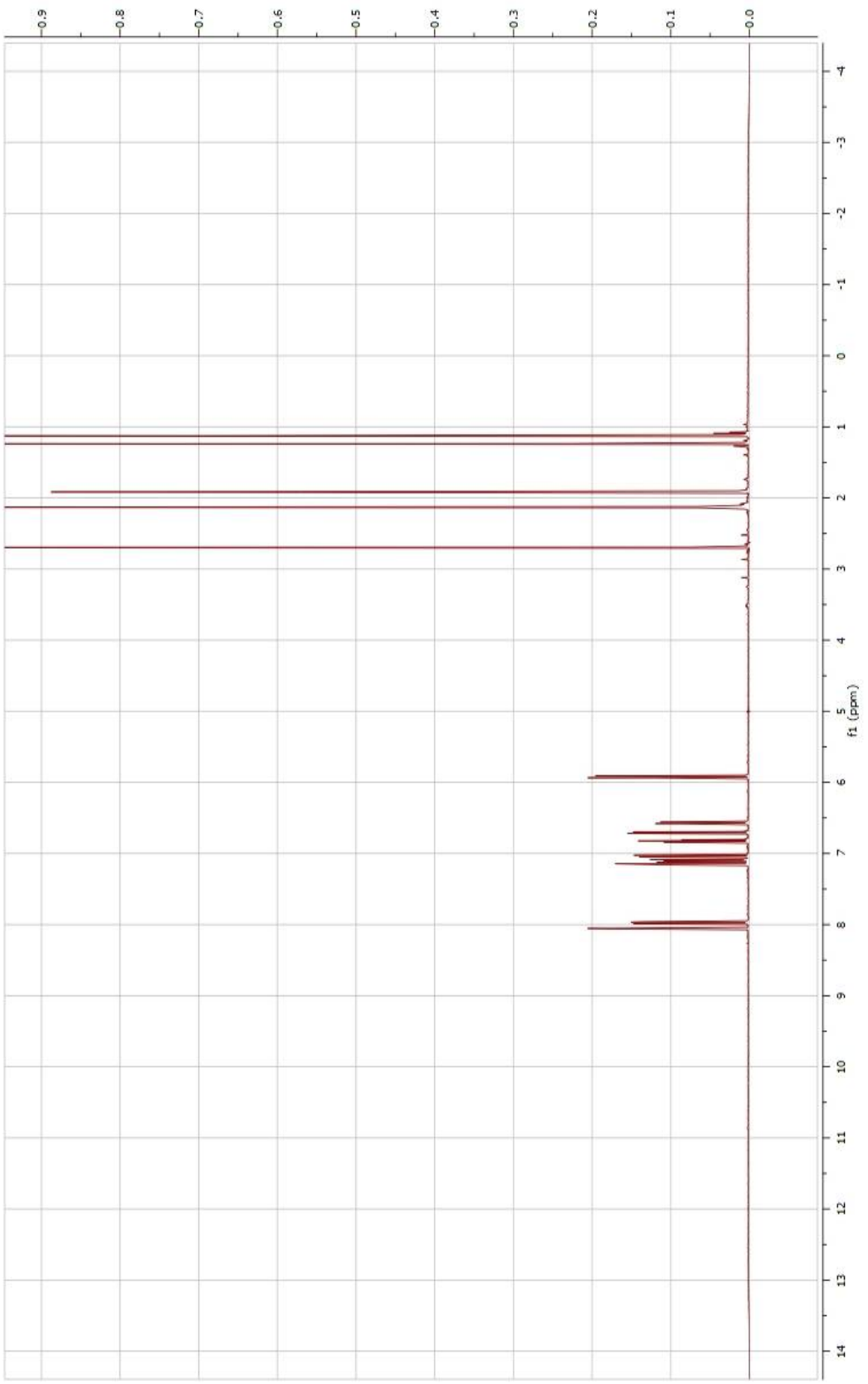

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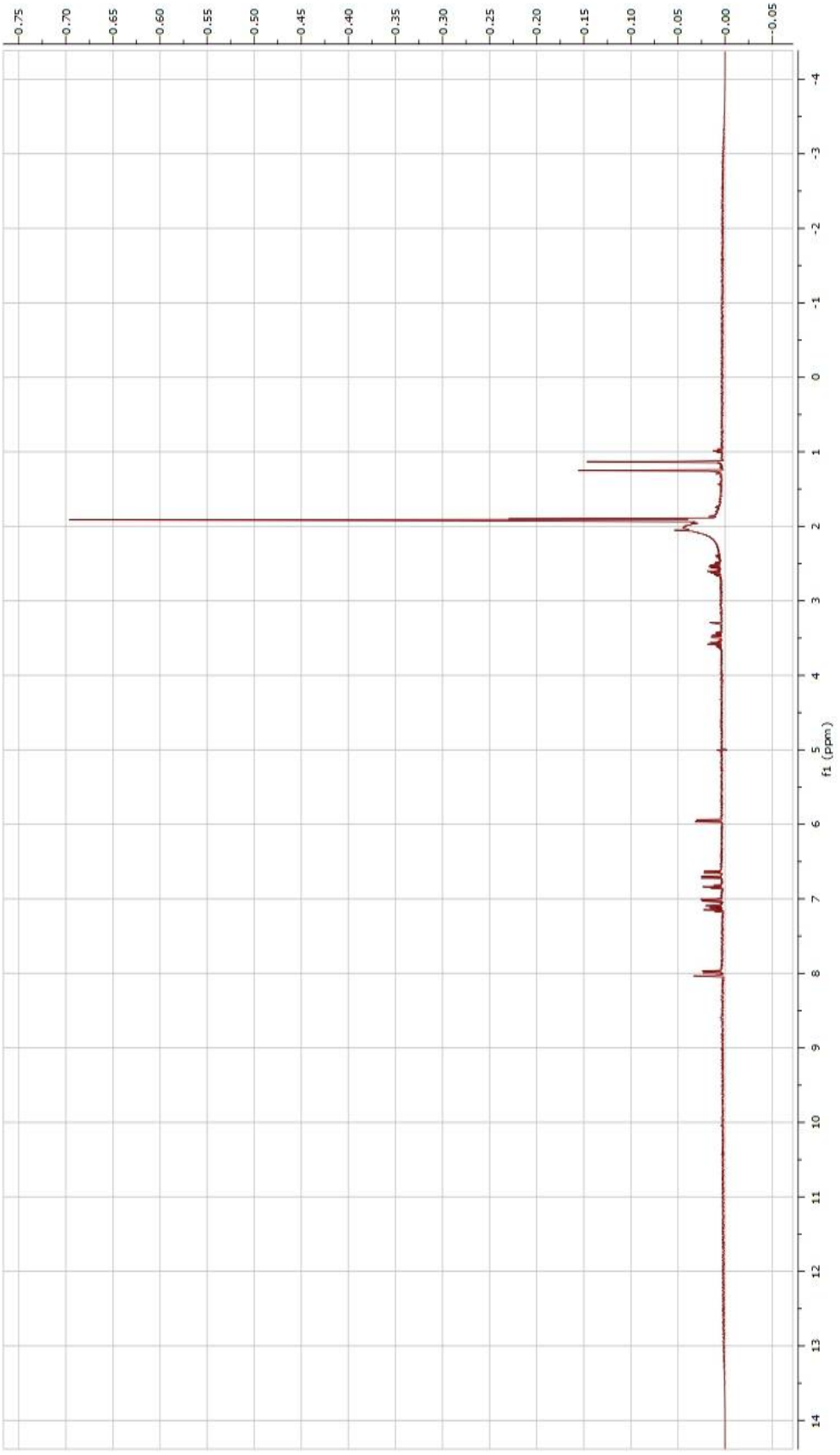

Poster Presentation

Optical switching of high-affinity biomolecules via photochromic dye groups

Cranfield UNIVERSITY Silsoe

Bernard D. Annan, Kal Karim, David C. Cullen
Institute of Bioscience and Technology, Cranfield University. Silsoe, Bedfordshire, MK45 4DT, UK

CRANFIELD BIOTECHNOLOGY CENTRE

Introduction

Figure 1. *Reactive lens (photochromic) glasses / sunglasses. A typical application of photochromism in the real word.*

- The high affinity and high specificity of antigen-antibody complexes provides grounds for many effective applications in biotechnology.
- Rapid reversible control of antigen-antibody affinity is a highly desired feature in applied medicinal and biotechnological fields.
- However it is often difficult to achieve complete dissociation and regeneration of antibody receptors without imparting irreversible damage.
- Our objective is to establish the practicality of "switchable affinity control by illumination" (*i.e.* switchable "on and of" antibodies) using photochromic dye molecules and to develop an understanding of the mechanism of action.
- Photochromic dye molecules are compounds that exist in typically two isomerised forms that possess differing physico-chemical properties. The two forms are inter-convertible by illumination at different wavelengths. This exceptional property realises the feasibility of a molecular optical switch.
- By covalently attaching photochromic dye molecules to antibodies, optical switching between the two isomeric forms is expected to reversibly distort the antibody protein structure thereby altering the antibody affinity (figure 3). For analytical applications – *i.e.* immunosensors, such affinity switchable antibodies would allow simple regeneration and reuse.

Method

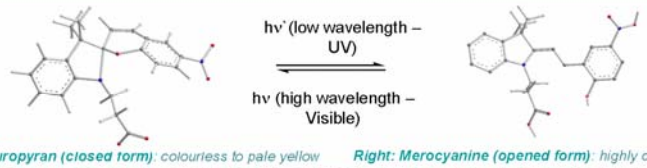

Figure 2. ***Left: Spiropyran (closed form)**: colourless to pale yellow* ***Right: Merocyanine (opened form)**: highly coloured (reddish)*
In non polar solvents spiropyran compounds (figure 2) exhibit normal photochromism and are colourless to pale yellow solutions becoming highly coloured when irradiated with U.V light and then reverting back to the colourless spiropyran form upon irradiation with visible light or via dark adaptation when left in the dark. The "closed" spiropyran is a neutral hydrophobic compound, when photo-isomerised the merocyanine compound is a highly coloured (e.g. which gives it its dye classification) zwitterionic compound and therefore more hydrophilic

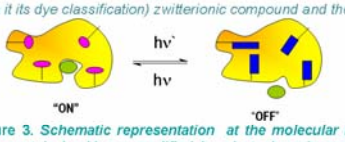

Figure 3. *Schematic representation at the molecular level of a protein backbone modified by photochromic groups. The different photoismoerisable forms changed by appropriate illumination, affect the structure and activity of the protein*

Photochromic Dye Synthesis

The Initial method to be employed involves the synthesis of a spiropyran dye (a type of photochromic dye). This involves the compounds 3-iodopropionic acid, 2,3,3-trimethylindolenine and 2-hydroxy-5-nitrobenzaldehyde (5-nitrosalicylaldehyde) to form the carboxylated spiropyran (figure 2). The dye is to be synthesised using a modified method of AIZWA, M., NAMBA, K., and SUZUKI, S. (1977).

Antibody Conjugation

The second step is the conjugation of the spiropyran dye to the antibody (in this study IgG Anti Atrazine recombinant single-chain antibody (scAb) fragments). The fragment contains the Fab region which is a region highly conserved of amino acids and variable region of amino acids.

Testing

The third step is to apply the coupled compounds in an Illuminant (light sensitive) assay such as a modified ELISA to asses the productivity of the proposed premise - switchable "on and of" antibodies via photochromic dye groups. Following an expected success, the compound will be incorporated into a bioactive system such as the Biacore (an optical biosensor) in which its system productivity will be assessed.

Results and future work

- The initial investigation of IgG Anti Atrazine recombinant single-chain antibody (scAb) fragments and Anti Fluorescein in an ELISA indicates that the fragments of Anti Atrazine works as well as the whole (complete) antibody (in this study, Anti Fluorescien) (results not presented). This indicates an advantage of low molecular weight of the chosen compound (Anti Atrazine) in a significantly good working order.
- The synthesised spiropyran dye purity will be indicated by TLC analysis, mass spectroscopy and melting point of the compound, the yield of the final end product will also be assessed. The Spiropyran dye and the complex formed (by the attachment to an antibody) in different solvents will also shows very different characteristic in each and the most appropriate will be known.

Figure 4. ***Microtitre plate during an ELISA assay**. These images are various steps of an ELISA assay, the "blue" is the observed colour change and the "yellow" indicated the end of the reaction when stopped.*

Conclusions

This initial study indicates that procedures for antibody analysis (ELISA) has been established and incorporation of the antibody - dye complex results will indicate whether the antibody affinity may be controlled by illumination at different wavelengths

Bernard Derek Annan, Tel: +44 (1525) 863729, b.d.annan.s04@cranfield.ac.uk

www.cranfield.ac.uk/ibst