

CRANFIELD UNIVERSITY

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**TOWARDS A REMOTE PORTABLE BIO-
AFFINITY SURFACE PLASMON RESONANCE
ANALYSER FOR ENVIRONMENTAL STEROIDAL
-POLLUTANTS**

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Supervisor

Dr. David C. Cullen

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Abstract

The widespread presence of chemicals with the capacity to disrupt the endocrine system in both wildlife and humans in our natural environment has increasingly become of major concern in the last ten years. Endocrine disrupting compounds (EDCs) are a group of compounds that pose a potentially dangerous and real threat to the health of both humans and wildlife. These substances can mimic or interfere with the biological pathways of natural endogenous signalling chemicals controlling the endocrine system (e.g. sex hormones). Endocrine disrupters are ubiquitous in water. The detection, monitoring and treatment of wastewaters and surface waters for EDCs would significantly help minimise the environmental burden imposed by these natural and synthetic compounds. To optimise such processes, an economical, *in-situ* or field-based detection technique for EDCs is required.

The research presented in this thesis describes the development of a portable surface plasmon resonance device for the detection of endocrine disrupters in wastewater and surface waters. The first two result chapters describe the construction, development and optimisation of the portable analyser and immunoassay protocol using anti-estrogenic antibodies. A novel approach for regenerating the SPR sensing surface was achieved by using Persil biological laundry liquid (1%). The developed immunoassay showed a working range between 0.2 - 7 μ g/L for Estrone-3-Gulcuronide (E₁3G) in buffer. The detection of 17 β - Estradiol (E₂) in buffer, synthetic wastewater and real wastewater samples was also carried out; the working range was 0.1 - 10 μ g/L; 0.3-7 μ g/L and 0.1-10 μ g/L respectively.

The second part of the thesis describes the synthesis and protocol development of a photo-chromic dye and its application to immuno-sensing systems *en route* to a reversible bio-affinity antibody for application to regenerating bio-sensing surfaces. This approach was to demonstrate the concept of remote regeneration of the active sensing surface for a portable optical sensor.

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IN MEMORY OF:

Uncle David, Uncle Alimamy, Dave and Frank

*“Found internally, certain compounds are important biological signals; found
in the environment, they become just so much noise”*

John A. Mclachan and Steven F .Arnold

“Technology is only an issue for those that were born before it was invented”

Anon

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Notations

ABTS	2,2'azino-bis-ethylbenzthiazoline-6-sulfonic acid
AFM	Atomic Force Microscope
APTS	3-aminopropyltrimethoxysilane
BPA	Bis-Phenol A
BSA	Bovine Serum Albumin
BSA-E ₂	Bovine Serum Albumin – Estradiol conjugate
BSA-FITC	Bovine serum albumin Fluorescein isothiocyanate
CMD	Carboxymethyl Dextran
E ₁	Estrone
E ₁ 3G	Estrone-3-Gulcuronide
E ₂	Estradiol
E ₃	Ethinylestradiol
EDAC	1-ethyl-3(3-dimethyl amino propyl) carbodiimide
EDC(s)	Endocrine disrupting chemical(s)
ELISA	Enzyme linked immunosorbent assay
FITC	Fluorescein isothiocyanate
HRP	Horseradish Peroxidase
IgG	Immunoglobulin G
LED	Light emitting diode
MC	Mereocyanine form
MES	2-[N-morpholino]ethanesulfonic acid
MEth	Mercaptoethanol
MUA	Mercaptoundecanoic acid
NHS	N-Hydroxy Succinimide

OD	Optical Density
OVA	Ovalbumin (chicken egg Protein)
OVA-E ₁ 3G	Ovalbumin – Estrone-3-Gulcuronide conjugate
PBS	Phosphate Buffered Saline Solution
PBST	Phosphate Buffered Saline and Tween 20 solution (0.5%)
RIU	Refractive Index Units
RO water	Reverse Osmosis
RU	Resonance Units
SP	Spiropyran dye
SPCOOH	Carboxylated spiropyran dye
SP-HRP	Spiropyran dye conjugated to HRP
SPR	Surface Plasmon Resonance
SP- α E ₂	Spiropyran dye conjugated to anti-17 β Estradiol
SP- α FITC	Spiropyran dye conjugated to anti-FITC Monoclonal Antibody
STP	Sewage treatment plants
TIRF	Total Internal Fluorescence
TISPR-1	Texas Instruments surface plasmon resonance sensor
TLC	Thin Layer Chromatography
TM	Trans-magnetic radiation
WWT	Waste Water Treatment
α E ₁ 3G	Anti-Estrone-3 Gulcuronide
α E ₂ or α 17 β E ₂	Anti-Estradiol monoclonal antibody
α FITC	Anti-FITC monoclonal antibody
α HGC	Human chronic gonadotrophin

1 Introduction and Literature Review

Chapter 1

Introduction and Literature Review

1.1 Background

The progression of industrialisation, new synthesis discoveries and processing of chemicals and drugs during the twentieth century, has shown the way for the introduction of many products and applications that have benefited the standard of living for humans globally (e.g. pesticides, contraception pills, plastics, food additives etc.). The increased consumption and the commercial and domestic demands on these synthetic products have also inversely led to our environment becoming increasingly polluted. According to R. Bhatt (*Bhatt, 2000*) more than 80,000 synthetic chemicals and metals are in commercial use in Europe alone. This is not taking into account those chemicals that have been banned and/or are no longer in production but are still being used or simply remain in the environment (e.g. DDT and PCBs). The toxicity or adverse long-term effects for many of these chemicals are often unknown and in many cases the data obtained is incomplete or insufficiently studied.

Over the last three decades there has been an increase in public and scientific concern on bio-hazardous chemical compounds present in the natural environment, especially those that have been observed to effect the reproductive and developmental cycle of wildlife and humans otherwise known as endocrine disrupting chemicals (EDCs). A concerted effort by the European Union, the Environmental Agencies of Great Britain and the USA (EA and EPA respectively) have encouraged and endorsed research into developing environmental quality standards, tests and monitoring tools to be in place as soon as possible. In the 1990s endocrine disrupters were one of the highest priority research topics for the EA and EPA and a detailed research strategy was developed to guide the placement of resources over several years (*Chase, 1998*).

Environmental pollutants have intensively been studied over the past thirty years. The focus has mainly been on the potential toxicity of compounds causing direct DNA damage (*McLachlan et al., 1996*). However, environmental estrogens do not alter genes but alter the way they express themselves therefore, current hazardous chemicals toxicity bioassays that rely on lethal dose toxicity exposure (e.g. LD₅₀ studies) are unsuitable and inadequate to measure or detect the extent of EDCs adverse effects in the environment.

Over twenty five years ago investigators were alerted and started expressing their concern of estrogenic effects of xenobiotic chemicals in the environment and their apparent effect on wildlife (*Mason et al., 1986; Nelson et al., 1978; Bitman et al., 1970*). More recently a highly acclaimed book by Dr Theo Colborn (*Colborn et al., 1997*) outlined the potential hazards that some of these chemicals may have on human and ecological well-being in the natural world (e.g. breast cancer and reproductive tract cancers, reduced male fertility, abnormality in sexual development etc). Many reports examining environmental disruption and their effects on human and wildlife have been documented in detail (*EPA, 1997; Toppari, 1996; Harrison et al., 1995*).

In recent years, endocrine disruption has been observed in wild and caged fish in rivers that received large inputs of pre-treated water from wastewater treatment plants. As a consequence increased concern has been raised about the presence and concentration of hormonal like compounds in wastewater effluent (*Huang et al., 2001*). It was found that the high concentration of environmental hormone mimics causing these adverse effects on fish populations were from natural and synthetically derived estrogenic compounds (e.g. 17 β Estradiol and ethinylestradiol) of which the main source was being produced by humans via excretion of urine and faeces.

With this in mind, an obvious need has been created for the development of analytical techniques that are suitable for screening and measuring the concentration of EDCs in the environment. Endocrine disrupting compounds are a ubiquitous diverse group of chemical compounds found in the environment. They are characterised by having low molecular weights and can have a physiological affect on cultured cells at very low concentrations (ng/L). The diversity of the chemical structures of estrogenic pollutant means that any development of an analytical device and/or monitoring technique would require full flexibility that could be applied to a range of different compounds as well as it being robust, cheap and functionally suitable for field and at source use.

Optical bio sensing is one of the oldest analytical techniques in immunosensing of which surface plasmon resonance technique is the most established. Texas Instrument has developed a miniaturised surface plasmon resonance sensor that is very robust and suitable for integration into original custom-made instruments. The sensor to date has been successfully used as an immunosensor for bio-molecular interactions (*Strong et al., 1999*). However, outside of the work presented in this thesis, the sensor has not yet been used as a portable analyser or for the detection of endocrine disrupting chemicals.

1.1.1 Thesis structure

The body of work presented in this thesis starts with a literature review of the current scientific knowledge of endocrine disrupting compounds as environmental pollutants, focussing on steroidal hormone mimics, in particular estrogenic mimicking compounds and their current methods of detection. Each results chapter begins with an introduction and general materials and methods that describe all experimental conditions used within the work pertaining to that chapter. The main body of results are divided into two parts. The first two results sections of the first part of the thesis investigate the development of an immunoassay and immuno-sensing applications for the detection of estrogenic compounds in buffered, synthetic and real wastewater samples and a novel approach for regenerating the biological sensing surface is described. The emphasis of the research was to devise a surface plasmon resonance bio-affinity analyser where the biological sensing surface could be remotely prepared and regenerated *in-situ* plus be amenable for use in the field.

The second part of the thesis describes the synthesis and experimental investigation of a spiropyran photo-chromic dye for the purpose of developing a reversible biological sensing surface. Using the dyes unique isomeric properties the development towards a reagentless bio-reversible antibody for regenerating bio-sensing surfaces was explored. This line of inquiry was in addition to demonstrate another approach for remote regeneration of the active sensing surface for a portable optical sensor in the field.

1.2 Literature Review

This chapter will review the current scientific evidence of endocrine disrupting compounds and their role as an environmental pollutant. The emphasis will be on endocrine disrupting chemicals that are estrogenic or anti-estrogenic in nature. Their mode of action, pathway, and physiological affects will be discussed. The chapter will present an overview of current analytical methods and strategies available for the detection of estrogenic effects and concentration of estrogens in environmental samples. Bio- sensing platforms and the use of surface plasmon resonance (SPR) as an analytical tool for micro-pollutants will also be discussed. Finally, photo-chromic dyes will be discussed in the context of applying them to the regeneration of bio sensing surfaces and true reversible immuno-sensing applications.

1.2.1 The problem

Scientific and public concern over the last ten years has focused on the hypothesis and realisation that certain chemicals present in the environment, both natural and anthropogenic, have the capability of causing a myriad of adverse effects through their modulation and interference of the endocrine systems in wildlife and humans. This intensified concern is due to increased incidences of hormone dependent diseases such as breast cancer, testicular cancer, endometrioses and birth defects. Environmental pollutants, with physiological effects similar to endogenous hormones, are generally known as hormone mimics and therefore raise concern due their ability to be recognised by the endocrine system's receptors in humans and wildlife.

1.2.2 Hormones and the endocrine system

Almost all living organisms has to a greater or lesser degree an intra and/or extra cellular homeostatic system that orchestrates and regulates the metabolic functions of the various organs and internal metabolic systems of an organism, be it mammals, non mammalian vertebrates, non vertebrates, insects or plants (*Environmental Agency, 1998; Stryer, 1988; Roberts, 1986*). In vertebrates, one of the main extracellular homeostatic systems is the endocrine system. The endocrine system is composed of a group of organs that secrete biological chemical substances into the blood whose collective role is to regulate the internal biological function of an animal. The definition of the word endocrine is “internal secretion” and the endocrine organs are internally secreting glands (i.e. ductless glands) that coordinate a chemical messenger pathway characterised by the production of biologically active substances. The biologically active substances are carried through the blood stream to be delivered at target organs instigating a cellular response (*Nishikawa et al., 1999; EPA, 1997; Stryer, 1988; Roberts, 1986*). The endocrine organs do not exist in functional isolation, but influence one another through cascading feedback loops and chemical signal interactions that characterises the highly complex and coordinated endocrine system (*Roberts, 1986*)

These biologically active substances are called hormones. They are active at very low concentrations (ng/mL or pg/mL i.e. ppb or ppt) and bind specifically to target receptor sites on cell surfaces or nuclear protein receptors. Once associated with their corresponding target site they exert important regulatory, growth or homeostatic effects. In wildlife, hormones regulate migration, mating behaviour, fat deposition, insect metamorphoses, hibernation, shedding of skin in snakes and shells in molluscs and shrimps. The endocrine system also controls the growth, metabolism and body functions (i.e. reproduction, fertility and mineral regeneration). In vertebrates, some of the major endocrine glands include the hypothalamus, pituitary, thyroid, parathyroid, pancreas, adrenals, pineal body and pars distils (*Roberts, 1986*). The secretion of hormones therefore aids both the regulation and metabolic reactions the body. The endocrine system includes a number of target organ feedback pathways in the central nervous system (CNS) that are involved in regulating a multitude of functions in maintaining the body. The complexity of the endocrine system with its cascading loops of hormone signals and responses lends itself to the possible interference of the system at many points (*Sesay et al., 2001; Arnold et al., 1997; Roberts, 1986*). Hence, offer many sites and organs where exogenous agents could potentially disrupt hormone function of the endocrine system. The complexity of the cellular processes involved with hormonal communication at any of these loci may inevitably lead to impaired hormonal control (i.e. the synthesis, storage/ release and transportation of hormones) (*EPA, 1997*).

Apart from the traditional hormones often referred to in higher vertebrate (including humans), hormones are present in invertebrates (e.g. ecdysone), plants (e.g. auxins) and fungi (e.g. gibberellins)(*Roberts, 1986*). Therefore, when environmental exogenous endocrine disrupters mimic or interfere with the action of endogenous hormones, their potential to influence the health of humans and exert significant ecological effects globally is a great possibility (*EPA, 1997*).

1.2.3 Endocrine/hormone disrupting compounds

Endocrine disrupting chemicals are natural or anthropogenic exogenous agents that can affect not only the individual but also populations and communities; hence whole ecosystems can be affected. The effect occurs by EDCs interfering with endogenous hormones in the body since these hormones are responsible for key regulatory processes in cell control and communication as well as reproduction and behaviour. An environmental endocrine or hormone disrupter is an exogenous agent that interferes with the synthesis, secretion and transport of endogenous hormones.

There has been much scientific deliberation about the exact meaning of an endocrine disrupting chemical. This is mainly due to partial knowledge of the pathways in which they interact where correlated data is incomplete (*EPA, 1997*). An approved international definition agreed by the International Program for Chemical Safety (IPCS), World Health Organisation (WHO), United Nation Environmental Protection (UNEP) and International Labour organisation (ILO) with Japan, Canada and USA, Organisation for Economic Co-operation and Development (OECD) and European Union (EU) states:

“A potential endocrine disrupter is an exogenous substance or mixture that possesses properties that might be expected to lead to endocrine disruption in an intact organism, or its progeny or sub population.”

In addition

“An endocrine disrupter is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny or sub populations.”

(European Commission, 1996)

There are certain substances that are able to interact with hormone receptors and thereby interfere with the synthesis and action of hormones in the body, consequently disrupting the physiological process under control of the endocrine system. This can occur in various ways, under certain conditions they may act as stimulatory agents and tumour promoters. Therefore, the dose and duration of exposure and stage (i.e. infancy, adolescence, etc.) are critical in the determination of assigned adverse effects. These effects may be reversible or irreversible, acute or chronic in expression.

The mode of action of endocrine disrupters or hormone mimics can be one that (i.) acts as a mimic or false hormone by binding to a receptor and causing a response such as estrogenic or androgenic effect (*Pöchlauer et al., 1998; Sonnenschein et al., 1998*), (ii.) inhibits binding to a receptor and prevents the natural hormone from producing the required response (i.e. agonistic effect) e.g. dioxins, (iii.) alters or interferes with the process of synthesising natural hormones and receptors or the processes that removes them from circulation or (iv.) modifies the hormone receptor levels triggering an abnormal response or action in the cell (*Environmental Agency, 1998; Sonnenschein et al., 1998*) (*Please refer to figure 1.1*).

It is also possible that EDCs could interfere with more than one hormonal system, simultaneously causing multiple responses. The mechanism of the endocrine system as mentioned above is a very complex one. Much of the research to date has focused on the disruption of the steroidal hormonal system where environmental polluting chemicals have been found to have an estrogenic, androgenic, and anti-estrogenic and/or anti-androgenic effect. The scientific debate has widened and potential effects on the other endocrine hormonal systems, such as the pituitary and thyroid hormones that influence growth, development and behaviour are now being considered and investigated.

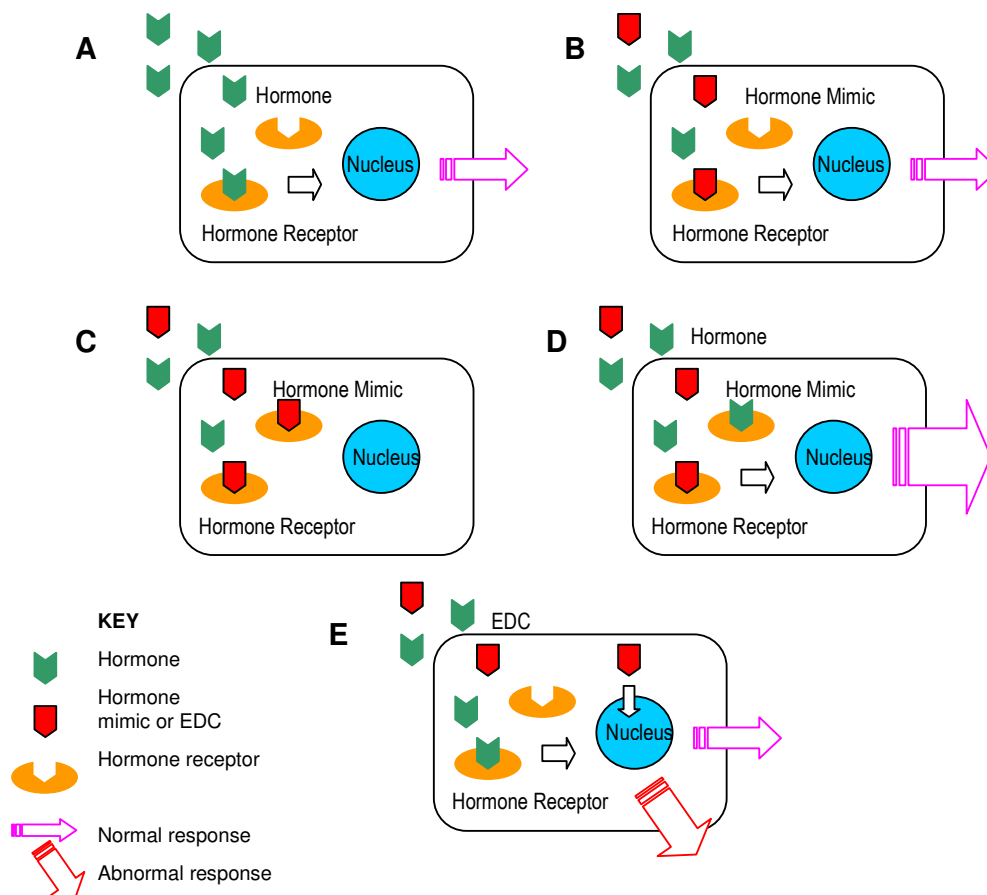


Figure 1.1: Endocrine Disrupting Processes. A) Normal hormonal and cellular response, B) Hormone mimic (EDC) eliciting an agonistic response, C) Receptor and EDC complex inhibiting a normal response i.e. antagonistic response. D) Hormone and hormone mimic displaying a synergistic response, E) EDC eliciting an abnormal cellular response independent from receptor binding mechanism.

The ranges of chemicals that have been reported to have endocrine disrupting effects are diverse and continue to expand as the number of studies increases. The number of possible EDCs is potentially great with over 80,000 chemicals currently in use and another 1000 new ones added each year (Sumpter, 1998). However, only a few chemical compounds have been tested for endocrine disrupting effects (Nagel et al., 1999). Some of these chemicals are likely to be distributed widely in the environment and in some cases are long lived and bio-accumulate in the tissues of plants and animals (please refer to Table 1.1).

Table 1.1: Categories and chemical examples of endocrine disrupting chemicals found in the natural environment

Compound Category	Examples	Uses	Environmental fate and -sinks	Mode of action
Naturally occurring chemical				
Phyto-estrogens	Coumentrol, geinistien, equol, isoflavones, ligans	Present in plant material, auxins are necessary for plant growth and propagation, and protection	Are eaten and/or decomposed and enter soil and water systems	Estrogenic and anti-estrogenic
Steroids	17 β Estradiol, Estrone, oestriol, progesterone, testosterone	Produced naturally by animals important for maturation, differentiation and reproduction	Released into the environment via excretion mainly via STW and is then discharge into watercourses (i.e. rivers. lakes, sea etc.)	Estrogenic, androgenic
Man-Made chemicals				
Synthetic produced pharmaceuticals	Ethinylestradiol, Methyleneestradiol, Diethylstilbestrol, Telengestrol acetate (MGA), Trenbolone, Zeranol	Produced as a contraceptive or for use in livestock farming	Mainly through mammal excretion and finds its way into watercourse	Estrogenic
Agriculturally and Industrially used chemicals				
Polyhalogenated organic compounds	Dioxins, polychlorinated biphenyls (PCB), PBBs, TCDD	Incineration by-products, and lubricants, (many are now banned or obsolete)	Bio-accumulative compounds still reside in aquatic animals and sediments	Anti estrogenic
Organo chlorinated pesticides	DDT, deildrin, lindane, HCB, Acteochlor, atrazine, endosulfan, 2,4,D, dicofol, prochloraz, malathion, simazine, kepone, chlordane	Used as an insecticide (many are now obsolete, banned or under restriction. However, still exist in the environment as they are known to bio accumulate.	Deliberately administered against insect infestation. As aerosols or powders. End up in soils and water course they bio accumulate in the food chain.	Estrogenic, anti-estrogenic, and anti-androgenic
Organo tin compounds	Tributyltin compounds (TBT), tetrabutyltin, triphenyltin	Anti-fouling agent, used on sailboat and ships to remove barnacles from the hull.	Discharged deliberately into estuaries, seas or oceans due to boat cleaning processes.	Anti estrogenic
Phenolic compounds, Alkylphenol and Alkylphenol ethoxylates	Nonylphenol, nonylphenol ethoxylate, bisphenol-A,	Used in the production for polymers, surfactants and anti viral preparations. Epoxy resins	Industrial waste, landfills, soils and WWT eventually end up in watercourses.	Estrogenic
Phthalates	Dibutyl phthalates (DBP), butylbenzyl phthalate (BBP), DEHP, Diisodecyl phthalate, diisononyl phthalate (DINP)	Plasticiser released	Industrial waste, landfills, soils, WWT and eventually in water courses	Estrogenic

1.2.3.a Overview of Environmental Estrogens

One of the major classes of endocrine chemical disrupters that have caused great concern are those compounds that mimic steroidal sex compounds and affect the reproductive system. These compounds have been called many generic names: eco-estrogens, environmental estrogens and xeno-estrogens, they nevertheless all refer to the same group of compounds. Natural estrogens are involved in the development and adult function of the female genital tract organs, neuron tissue, endocrine tissue and the mammary glands. Males also have cells that are responsive to estrogen (*Diel et al., 2002*). There are a plethora of compounds that have been implicated in having endocrine disruptive estrogenic action. These compounds are either naturally derived chemicals found in plants (phyto-estrogens), fungi (myco-estrogens) or man made (xeno-estrogens) (*McLachlan et al., 1996; Arnold et al., 1996b*).

Endogenous estrogens are steroidal hormones and are derived from the aromatisation of cholesterol via testosterone. That are produced in the ovaries of females and the testes of males in response to feedback signals from the brain and other organs (*Arnold et al., 1996a*). In mammals these hormones are transported in the blood by sex binding hormone globulins (SHBG) found in the plasma that have a high affinity for hormones and determines the circulation levels of free and bound estrogens in blood plasma where they are transported to the cells of target organs (e.g. uterus). Steroidal compounds are highly hydrophobic compounds and can cross the cell membrane very easily and unaided. They act by binding to an estrogen receptor protein. The steroid / receptor complex binds to a specific area on the DNA known as the estrogen response elements (ERE). The complex can either activate or repress gene expression or alter the level at which the gene is expressed. The end result is a change in the cells program (*Arnold et al., 1996b*).

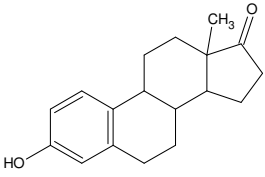
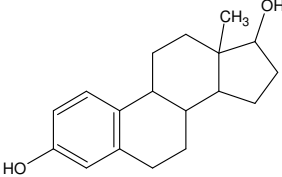
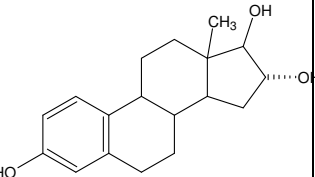
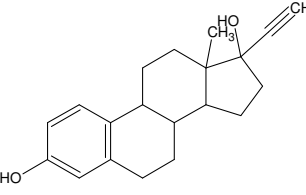
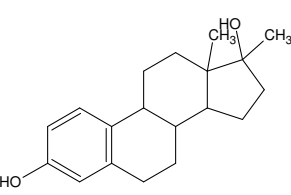
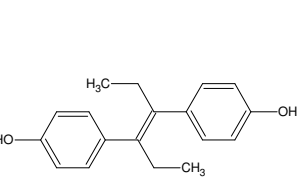
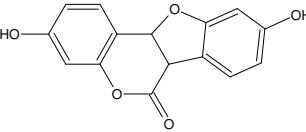
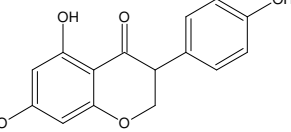
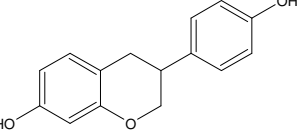
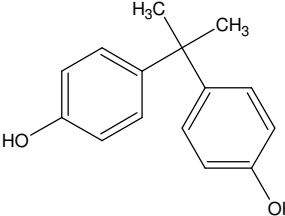
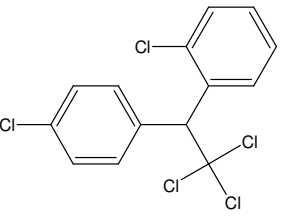
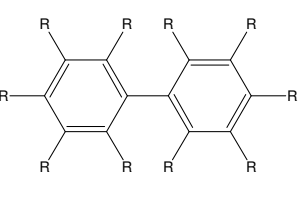
Table 1.2: Estrogen sensitive tissue and cells
(Deil et al. 2002)

Classic Targets	Non-classic target
Ovary	Kidney
Vagina	Islets of langerhans
Uterus	Liver
Mammary glands	Bone
Adrenal glands	Cardiovascular system
Prostate	Macrophages
Piturity Gland	Thymocytes
Hypothalamus	Lymphoid cells
	Endothelial cells
	Ostoblastic cells
	Gila cells
	Schwann cells
	Adipose tissue

Estrogen hormones regulate the reproductive cycle by causing stimulation, cell proliferation and growth of reproductive organs to maintain pregnancy and by influencing secondary sexual characteristics (Kaplan, 1999). The affinity of endogenous steroids is high and the dissociation is low therefore, the levels of hormone required in circulation to elicit a response are at low concentrations. Only some cells contain these hormone receptors and hence can respond to estrogen and their mimics (please refer to table 1.2). It was quite an unexpected scientific breakthrough when many compounds with very different chemical structures from the steroidal chemical structure were able to elicit an antagonistic or agonistic estrogenic response (Soto et al., 1995). Table 1.3 illustrates the broad range of identified estrogenic compounds and their different chemical structure compared to natural steroids.

Table 1.3: Chemical structures of animal and plant based estrogens and xeno-estrogens

(Adapted from Hock, and Seifert, 1998)

Endogenous Estrogens		
		
Estrone	17β Estradiol	Estriol
Synthetic Estrogens		
		
Ethinylestradiol	Methyleneestradiol	Diethylstilbestrol
Phytosterogens (plant estrogens)		
		
Genstein	Coumestrol	Equol
Xenoestrogen (hormone mimics)		
		
Bisphenol A	DDT	PCB (R= H or Cl)

1.2.3.b Estrogenic pollutants an environmental problem

To date most environmentally polluting chemicals of interest and of concern are low molecular weight compounds (<300kD) and some consequently mimic hormones, particularly steroidal hormones. For most chemicals, whether natural or synthetic, independently of how they are used or disposed of (i.e. sprayed on agricultural land, in landfill from degrading rubbish, at water treatment works via drains) will eventually enter the aquatic system. The common route of exposure for terrestrial and aquatic wildlife to environmental pollutants is by contact with contaminated surface water. Therefore, the aquatic environment becomes the ultimate sink for environmentally polluting chemicals (*Sumpter, 1998*). It is therefore, no coincidence that many well documented case studies of endocrine disruption caused by environmental pollution have been in animals whose habitats are in aquatic environments (e.g. alligators, otters and fish) (*Guillette et al., 1994; Purdom et al., 1994; Mason et al., 1986*) or connected via their food web or feeding habitats (e.g. fish-eating birds). It is an ecological conjecture that these animals would be highly sensitive to these chemicals as they would receive the highest level of exposure and the bio-accumulative effect in their diets would warrant severe affliction.

1.2.3.c Steroidal hormones as endocrine disruptors

At the beginning of the 1970s Tabak et al described natural estrogens as potential environmental pollutants (*Hohsaka et al., 1994; Tabak et al., 1970*). This hypothetical proposition has been proved (*Shore et al., 1993*). Mammals excrete estrogenic hormones as sulphates or glucuronide conjugates in urine and in un-metabolised forms in faeces. The conjugated hormones are inactive but are often converted back into their original active form by biological processes in waste water treatment (WWT) by oxidase or sulphatase enzymes (*Huang et al., 2001*). EDCs can enter surface water by a variety of mechanisms including direct discharge from sewage treatment plant (STP) effluents, agricultural drains into streams and rivers and overland in rain runoff. Once in the waterway a series of processes such as dilution, photolysis, biodegradation and sorption into bed sediments takes place. Generally EDCs have low polarity, which tend to make them accumulate in the sediment where their retention time is considerably long. Therefore, alluvial sediment can act as a potential secondary source for EDCs within fluvial systems. This is due to sediment being transported to other areas and eventually the absorbed pollutant being released by diffusion across the sediment water interface or through sediment re-suspension at high water velocity (*Petrovic et al., 2001; Johnson et al., 1998*).

1.2.4 Evidence of Endocrine Disruption in the Environment

1.2.4.a Evidence in wildlife

Observation of changes in the reproductive system of wildlife caused by certain environmentally polluting chemicals has been well documented over the past forty years (www.environment-agency.gov.uk). One of the first observations was the declining population and unsuccessful egg nesting of the American bald eagle in 1947. It was noted at the time that 80% of the bald eagles were sterile (*Broley, 1958*). Rachel Carlson, who wrote “Silent Spring” in the 1950s, highlighted the effect of indiscriminate pesticide usage of DDT and other related chlorinated pesticides on the population of songbirds. Scientists later found that these birds were laying eggs with thin shells, hence making them prone to breaking, unsuccessful nesting and embryo mortality due to exposure to these chlorinated insecticides (e.g. DDT, Kapone) (*Colborn et al., 1997; Plamiter et al., 1978*). In addition the feminising effect of the surviving chicks led to the observation of female-female pairing (*Fry et al., 2003; Hunt et al., 1977*). A comprehensive review on birds and pesticides can be found in (*Fry, 1995*).

Endocrine disrupting effects have been studied and observed on aquatic wildlife species. In the UK it was identified that female dog whelks around the coast were showing male characteristics (i.e. imposex) and this physical deformation prevented them from laying eggs. It was then identified that organo-tin compounds (e.g. tributyl tin: TBT), used as anti fouling agent for boats were the cause. By the late 1980s TBT-based paints were banned for use in smaller boats and by the end of 2003 were banned altogether (*EPA, 1997*). In the mid 1990s dog whelks were observed to have shown some signs of recovery.

In the 1980s, British scientists were alerted to the feminisation of the male roach fish (*Rutilus rutilus*) on the river Lea in north London. Work carried out by Purdom in 1994 and Harris in 1996 noted that fish caught in lagoons below sewage treatment works (STW) were showing hermaphroditic characteristics (*Harries et al., 1996; Purdom et al., 1994*). A hypothesis was put forward that estrogenic chemical agents were being introduced into the watercourse and were causing this sex change phenomenon. In addition, the likely source of estrogenic EDC pollutants was from the widespread use of the contraceptive pill, and the subsequent release of ethinylestradiol and alkylphenol-polyethoxylates (APEs) originating from the biodegradation of surfactants and

detergents via the sewage treatment works and wool scouring mills. Further studies have supported this theory and found that the most abundant compounds present were Estradiol and ethinylestradiol both derived from human waste (*Harries et al., 1997; Harries et al., 1996; Nimrod et al., 1996; Purdom et al., 1994*).

Vitellogenin is a phospholipoprotein that is synthesised in the liver of female oviparous vertebrates and is naturally induced in females as a response to the presences of estrogens (e.g. 17β Estradiol) (*Nimrod et al., 1996*). Vitellogenin is absorbed into the blood stream via the liver where it is utilized by the ovaries to be transformed into two major types of proteins: lipovitellins and phosvitins (*EPA, 1997*). To determine the effect of estrogenic compounds in natural surface waters, vitellogenin levels in the serum of male fish species have been used as a biomarker (*Andersen et al., 1999; Sherry et al., 1999; Hansen et al., 1998*) (*See section 1.2.5.1*).

Since 1994 further studies on caged fish placed down stream from STW have further confirmed the above hypothesis. It has also been observed that male fish have displayed a reduction in the size of their testes and the vitellogenin levels in blood serum of male fish (*Oncorhynchus mykiss*) was found to be 500-10,000 times higher than expected in sewage outflow exposed areas (*Hansen et al., 1998; Jobling et al., 1996; Sumpter et al., 1995*). This effect decreased with distance from the receiving outlet of the sewage treated water out flow (*Harries et al., 1996*). Desbrow et al (1998) showed that fish exposed to ethinylestradiol gave an estrogenic response at exposure levels less than 1ppt (*Desbrow et al., 1998*).

The populations of frogs, toads and salamanders have declined dramatically worldwide (*EPA, 1997*). The University of California, Berkeley conducted a research into the decline of wild leopard frogs (*Rana pipiens*) across the Midwest corn-growing belt of North America. They concluded that Atrazine, a common herbicide used to control weeds in crop fields, was having a devastating effect on frogs at exposure levels <0.1ppb. This level is 30 times lower than the current US EPA levels allowed (3ppb in drinking water and 12ppb exposure limit for aquatic life (*EPA, 1997*)). In the wild, 80% of the male Leopold frogs were showing female characteristics i.e. reduced sized vocal chords and a 10-fold decrease in testosterone levels (lower than the normal level of female frogs). In all of the sites monitored, it was observed that only 16% of the wild frogs displayed genital normality. Further laboratory test showed that frogs raised in tanks and exposed to Atrazine (<0.1ppb) developed eggs in their testes, hence, showing hermaphroditic characteristics (*Hayes et al., 2002*).

A well-documented example of endocrine disruption in wildlife caused by environmental pollution was from a chemical spill in 1980 into Lake Apopka in the Florida everglades. The chemical spill contained high levels of DDT, DDE, and related compounds, dichlorobenzophenone, dicofol, and chlorinezilate. Consequently, many reports and observations followed detailing a variety of endocrine-related abnormalities. Resident male alligators were emasculated, as juvenile males had phalluses one half to one fourth of their normal size, they also showed decreased levels of testosterone (*Guillette et al., 1994*). It was also observed that 80-95% of eggs failed to hatch compared to normal failed hatch levels of 5-20%. Of those that hatch there was a mortality rate of 50% in the first two weeks. This is 10 times higher than those in nests of unaffected areas. Of the remaining hatchlings, female alligators had twice the amount of estrogen in their blood than normal and males had almost no testosterone (*Bhatt, 2000*). It was also noted that 90% of the population of juvenile alligators on lake Apopka had declined between 1980-1987 (*Woodward et al., 1993*). Red eared turtles were also observed to be emasculated (*Tyler et al., 1998; EPA, 1997*). *Tyler et al. (1998)* and *Vos et al. (2001)* both give a comprehensive critical review of all aspects of endocrine disruption evidence in wild life (*Tyler et al., 1998*) and special reference to the European situation (*Vos et al., 2000*).

In mammals, there have been several reports all indicating endocrine disruption in the wild. High concentrations of lipophilic compounds (i.e. mercury, p,p'-DDE and PCB) have been found in the tissue of panthers and raccoons and otters. The male Florida panther has been observed to have lower testosterone to estrogen levels than normal, making males more feminine and females more masculine. Male panthers were also observed having an increased incidence of undescended testes (cryptorchidism). This was attributed to the anti-androgenic effect of DDE (*Facemire et al., 2003*). The decline of British otters is also linked to the presence of organo-chlorines and estrogenic endocrine disruption (*Mason et al., 1986*).

It is very difficult to ascertain whether the long-term viability of wildlife populations is being affected by EDC pollution from the examples given in the literature. As exposure to sub-lethal concentration of EDC may not cause sudden population decline or obvious phenotype altering characteristic and therefore, may not be detected and ultimately affecting population levels years later. Predisposed susceptibility traits of sub progeny to certain illnesses and diseases due to EDC may also make it difficult to decipher the actual link to exposure. It is therefore, difficult to separate the effects of endocrine disrupting compounds from other factors such as habitat loss and other pollutants (*Kaplan, 1999*). The examples given above show the diversity of the problem and the potential of EDCs having widespread impact on habitats.

Endocrine disruption effects by EDCs on wildlife has been reviewed at length elsewhere (*Ameral Mendes, 2002; Vos et al., 2000; Sumpter, 1998*).

1.2.4.b Evidence in Human health

The first reported observation of human health effects due to exposure to endocrine disrupting chemicals was in the 1940s, when aviation crop-dusters handling DDT were found to have reduced sperm counts. Since then, there have been many reports linking DDT to breast cancer, (*EPA, 1997; Dewailly et al., 1994; Colborn et al., 1993*). In 1975, a chemical spill of Kapone an organo-chlorinated pesticide, resulted in low sperm counts in men that were exposed (*McLachlan et al., 1996; Plamiter et al., 1978*). Workers at a plant producing Kapone were reported to have had low sperm counts and to have lost their libido that consequently lead to impotency (*Sonnenschein et al., 1998*).

A powerful synthetic estrogen called Diethylstilbestrol (DES) was given to women from 1947 to 1971 to prevent miscarriages. It was observed in 1970 that DES was associated with a rare form of vaginal cancer called clear-cell adenocarcinoma that caused cellular changes in the vagina and/or fallopian tubes and structural changes in the uterus of adolescent daughters of women who had taken DES during pregnancy. Male children were also affected, displaying increased incidence of un-descended testes and genital tract abnormalities. Although this exposure was through pharmaceutical prescription, it was the first documented example of a human “transplental” carcinogen and scientific evidence that compounds that can mimic estrogens could have a devastating effect on the reproductive organs and morphology in humans. It was observed that DES was a far more potent estrogen than the natural 17β -Estradiol as DES has little affinity to human serum proteins (i.e. SHBG). Therefore, more is available for receptor activation (*McLachlan et al., 1996*). Since 1970 extensive research has been conducted on the effect of DES in mammals and human (*Gill et al., 1979*).

Dioxins, which are by-products of incineration processes, are highly toxic environmental pollutants. They are capable of lowering androgens levels and affecting the amount of thyroid hormones produced. Although not an estrogenic mimic it is able to block estrogen receptors and affect insulin levels and glucocorticoid secretion via the adrenals (*Petterson et al., 1993*). It was also observed that men exposed to agent orange which is a herbicide containing dioxin, had high incidence of fathering children with birth defects (*Bhatt, 2000*).

Excluding the few examples given above, there is only a tentative link of evidence implicating adverse effects of environmental estrogen mimics (i.e. exogenous hormones and their mimics present at low concentrations in the natural environment) to human health, such as low sperm counts, increased testicular and prostate cancer, increased incidents of male genital abnormalities; cryptorchidism and hypospadias (*Toppari, 1996*) and breast cancer (*Colborn et al., 1997*). Adverse health effect in humans operating via endocrine disruption has not yet been conclusively established (*Baker, 2001; EPA, 1997*). Many comprehensive reviews on human health and endocrine disrupters has been covered (*Ameral Mendes, 2002; Bhatt, 2000; Toppari, 1996*).

Attempts to link possible human health risks to hormone mimics is complicated by the fact that the individual potency to endocrine receptors in the body is very low (*Kortenkamp et al., 1999*), plus the average dietary exposure to xeno-estrogen would have to be at least 100,000 times higher to be able to elicit a receptor response, which is considered to be an insufficient amount to evoke an adverse effect in adults (*Safe, 1995; Safe et al., 1991*). Other studies suggest that endocrine disrupters may not significantly contribute to the development of hormone dependent disease or compromise reproductive fitness in humans (*Zacharewski, 1998*). However, these studies do not take into account the affinity of these compounds to serum protein (bio-availability), synergism of these compounds when present together at low concentrations (*Kortenkamp et al., 1999*) nor the ability of a developing foetus or the young to regulate sufficiently the burden of these exogenous endocrine disrupting chemicals when exposed. Endocrine disrupting chemicals are often highly lipophilic and bio-accumulative. High concentration can be found in breast milk and can pass the placental membrane to the unborn foetus very easily. Children exposure risks to these lipophilic compounds could be 10-40 times greater than the daily exposure of an adult (*WHO, 1989*). In conclusion, there are still a lot of research questions and data gaps to be answered and filled (*EPA, 1997*).

1.2.5 Legislation and environmental monitoring of EDC

The chemical industry is the third largest manufacturing industry in Europe. There are at least 100,000 different substances registered in the EU market of which 10,000 are marketed in volumes of more than 10 tonnes [EC Com (2001) 262]. The trade surplus is more than €41 billion per year. Since 1998, over 2,700 new substances have been made. Our current dependences and use of chemicals in our everyday life has had a knock on effect on our environment. The concerns of these industrialised synthetic chemicals were mainly due to their real or potential carcinogenic or teratogenic effects on wildlife and

humans. As most of these contaminants will eventually find themselves or their derivatives in natural watercourses, public concern for the hazards they pose to human health through contamination of drinking water supplies and the presences of chemicals in foodstuffs has led to stringent legislation being introduced, to monitor and control the release of these potential environmental hazardous pollutants.

1.2.5.a Past and Present Legislative Instruments

The European Union has been concerned about environmental pollutants since the early 1970 and has a range of policy instruments available to the community in the form of directives. In 1980 the European Union passed a drinking water directive [80/778/EEC] and its amendment [98/83/EC] setting the “Standards for the quality of water for human consumption, irrespective of the water source” (*Gardiner et al., 1984*). It states that the maximum concentration of any individual group of synthetic compounds (e.g. pesticides) should not exceed 0.1µg/L and the total combined pesticides and related compounds should not exceed 0.5µg/L. In the United Kingdom this directive is monitored and enforced by the environmental agency (*European Community, 1980*). Under the European dangerous substance directive (76/464/EEC), and its daughter directives and amendments, the EC proposed a priority candidate list of substances listing 140 compounds. The UK Government also identified 21 dangerous substances collectively classified as the “Red List”(*Baskeyfield, 2004*).

In October 2000 the European parliament and council established a framework for community action in the field of water policy. Altogether 11 council directives all pertaining to aquatic environment of the community including the council directive on pollution caused by certain dangerous substance discharged [76/464/EEC] and drinking water directive [80/778/EU] with their measures were to be either replaced, harmonised and/or further developed by directive [2000/60/EC]. The new community action water policy directive is aimed to prioritise the progressive reduction of priority hazardous substances by cessation of, or phasing out of, discharges, emissions, and losses within 20 years after the adoption of the policy (*Hellinga et al., 1998*). It represents a cohesive and transparent attempt to disentangled and demystify the legislative and monitoring instruments available to the community for adoption and enforcement. It also brings into account a classification of substances that have been identified as endocrine disruptors; *Table 1.4* gives a list of categories of the main pollutants.

Table 1.4: European Community priority list of chemicals and groups of chemical indicated to be endocrine disruptors

INDICATIVE LIST OF THE MAIN POLLUTANTS

(used for priority sub substance list in 2000/60/EC amendments and reclassification of 76/464/EEC list)

Organo-halogens, Organo-phosphorus, Organo- tins

Substances and preparations, or breakdown products of such which have been proved to have carcinogenic, mutagenic properties or properties which may affect steroidogenic, thyroid, reproduction or other endocrine-related function in/or via aquatic environments.

Persistent hydro carbon and bio accumulative agents and toxic substances

Cyanide

Metal compounds

Arsenic compounds

Biocides, plant protection products

Materials in suspensions

Substances that may cause eutrophication (i.e. nitrate, phosphates)

Substance that may affect the oxygen balance in the aquatic environment (BOD, COD, TOD)

The water policy included a list of priority substances selected amongst those that represent a significant risk to or via aquatic environments. The first list drawn up consists of 33 priority substances or groups of substances that were selected on the basis of the combined monitoring-based and modelling-based priority setting scheme (COMMPS). The list includes all of the chemicals identified in the directive [76/464/EEC] and it also amends and replaces those that appear in list 1 of Council directive [80/778/EU]. Altogether, this list included approximately 140 chemicals of which all of UK “red list” chemicals appear.

1.2.5.b EU Community strategy framework pertaining to endocrine disrupters

In 1996 a European workshop in Weybridge, UK agreed on an integrated plan for future research and monitoring activities on the impact of endocrine disrupters on human health and wildlife (*European Commission, 1996*). It was supported by many international agencies and international and national governments i.e. European Commission (EC), European Environmental Agency, the World Health Organization's European Centre for Environment and Health (WHO-ECEH), the Organization for Economic Cooperation and Development (OECD), national environmental agencies from England, Germany, Sweden, and the Netherlands, European Chemical Industry Council (CEFI), European Centre for Ecotoxicology and Toxicology of Chemicals (ECTOC), and members from the USA and Japan. Here many definitions and agreements on what substances constitutes in being an endocrine disrupter and a proposal of a working priority strategy for exchanging and filling information gaps in chemical data was discussed and agreed upon.

In 1998 the European parliament adopted a resolution calling upon the commission to take action in this area to improve the legislation framework and reinforce research effort and disseminating information to the public. This fed through to the newly drawn up water quality policy [2000/60/EC]. The commission in 1999 adopted a community strategy on endocrine disruptors [Com (1999) 706] that was inline with the precautionary principle. This set out the actions to be undertaken on the potential environmental and health impacts of endocrine disruption. The European commission communication report [com (1999) 706] was updated in 2001 with the commission adopting a list of a range of priority substance suspected of interfering with the hormonal system of humans and wildlife [com (2001) 262]. The list adopted was produced in a report entitled "Towards the establishment of a priority list of substances for further evaluation of their role in endocrine disruption". The report identified 553 man made substances and 9 synthetic/natural hormones. The report set out a priority list of action; timeframes and grouping in order to further evaluate the role of these substances in endocrine disruptions. (*Report and detailed information can be found on the European commission EDC website <http://europa.eu.int>*).

The priority framework is set out in three phases: short term: 1-2 year, mid term 2-4 years and long-term >4 years. Priority in the short term, is given to conducting an in depth study of 12 candidate substances which includes up to date EDC evidence, dose-potency, timing and synergy consideration and comparison with non endocrine disrupting toxic effect and quantitative

exposure assessment where appropriate. Nine of these chemicals are industrial chemicals and the other three are synthetic or natural hormones.

A concerted effort by international agencies and governments has led to greater information transfer and agreements to research initiatives. In 1999, the European Union and USA agreed to an EU-USA science and technology agreement. This identified a common priority research program and subsequently liberated funding for research within Europe and US. The global disruption research innovatory initiative is being supported by the European Union, WHO and IPCE. (*Damstra et al., 2002*).

The EU continues to participate and collaborated with the OECD and USA in what is known as the endocrine disrupter testing and assessment task force (EDTA). The EDTA was set up in 1998 with the goal of developing and validating agreed new and established test methods and test strategy for endocrine disrupters [Com (1999) 706]. These tests will harmonise any current research methods and tests that will be recognised and accepted by the different international and national organisation. It was agreed and estimated that in 2000, test methods for human health would be available in 2002, while test for environmental effect would be available in the timeframe from 2003 to 2005. Under the 5th community framework programme for R&D (1999-2002) research into endocrine disruption was prioritised. A dedicated call for research proposals on health and environmental impact of endocrine disrupters was announced in May 2001 and was given a budgetary envelop of €20M.

1.2.5.c Legislation action on endocrine disruption

Many of the chemicals that have been listed in the priority candidate list of the [79/434/EEC] dangerous substances and amended [80/60/EEC] drinking water act have also proved to be estrogenic in nature. This has meant that inclusion directly or indirectly into different existing legislative policies and directives would be coordinated. Therefore, EDCs will be included into the newly drawn up water policy and also in the recent white paper on “A strategy for a future chemical policy” as well as in the proposed revision of the general product safety directive.

1.3 Analytical Techniques for the determination of EDC

To date most of the techniques that are being developed are specifically for EDCs that affect the sexual reproductive system (i.e. xeno-estrogens). The following section will concentrate on these test methods. A concerted effort to identify, screen and rank endocrine disrupting compounds has started. As mentioned above OECD, EU and other international institutes are currently evaluating and harmonising current methods and analytical techniques for endocrine disrupters (www.oecd.org/ehs/ENDOCRIN.htm). The proposed strategy for identifying and screening EDC is to use a tiered approach where chemicals are prioritised, tiered, and screened (tier 1) and then tested (tier 2; i.e. test batteries). The analysis of EDCs falls into two categories:

1. Determination of chemicals as endocrine disrupters, these techniques often give information on effect and level of toxicity.
2. Quantitative measurement of concentration of known endocrine disrupting compound (e.g. whether in environmental sample or in industrial samples).

Many exogenous estrogens have been considered safe because they exhibit very low acute toxicity. However, these same compounds can show estrogenic activity at concentrations many magnitudes lower than their level of lethal toxicity (*Nagel et al., 1999*).

Endocrine disrupters can elicit effects through receptor-mediated mechanisms of action of sex steroid as well as receptor independent mechanisms that may involve steroidal transport, steroidal synthesis and interactions with target cell membranes. It is for this reason why a comprehensive evaluation of an EDC requires a battery of complimentary *in vitro* and *in vivo* assays that represent these mechanism activity pathways.

1.3.1 Endocrine disrupting effects based methods

These analysis methods can be divided into two sections: *in vivo* and *in vitro* analysis techniques. The advantages and disadvantages of *in vitro* and *in vivo* assays as well as emerging methodologies have recently been reviewed (Diel *et al.*, 2002; Eisenbrand *et al.*, 2002; Gray *et al.*, 2002; Zacharewski, 1998). A brief account of the well established techniques and variation of them are given in this section.

1.3.1.a In vivo assays

Many tests on mammals have been developed to specifically identify whether a chemical is an EDC or not and are able to give additional information. Over the last 60 years there have been a diverse numbers of animal models and assays that have been used to measure reproductive effects of chemicals (Sonnenschein *et al.*, 1998). The rodent uterotrophic assays and the vaginal cell cornification assays are two classic and well established techniques for assessing estrogenic substances (Baker, 2001).

The uterotrophic assay measures the increased weight of the uterus of immature or ovariectomised rodents post exposed to the compound of interest. This assay is sensitive and can measure additive effects of multiple estrogens. The assay can also produce false positives as a result of exposure to androgens or progestrogens (Folmer *et al.*, 2002). The vaginal cornification assay detects the histological changes in the vaginal epithelium in ovariectomized rodents. It is believed to be a definitive *in vivo* test for identifying estrogenic substances or complex mixtures as only compounds considered to be estrogenic and can induce cell proliferation. This assay has the advantage of being relatively simple and the same animal can be repeatedly used, providing that the test compound does not bio-accumulate. The disadvantage is that the assay is by large a qualitative technique and although optimisation has been introduced to address this issue a large number of animals are required to ensure repetitive accurate results (Zacharewski, 1998). The most widely used assay for androgenicity is the Hershberger assay. This assay detects the ability of a compound to elicit agonistic or antagonistic effects at the androgen receptor. Traditionally the assay is performed on castrated male rodents and measures the increased tissue weight of the ventral prostate and seminal vesicles of the testis (Gray *et al.*, 2002).

General disadvantages of using rodent models is that rodents do not produce sex hormone binding globulin (SHGB) which as mentioned before in Section 1.2.2.a is important in regulating the level of free estrogen present in the blood. Therefore, rodent models are often oversensitive to certain compounds that have high affinity to these proteins.

The uterotrophic and Hershberger assay, both short term *in-vivo* screening tests are currently in the process of being validated by the OECD to develop two test guidelines for identification of hormonal disruption (*Meyer, 2003; OECD, 2001a; OECD, 2001b*). The evaluation of four variations of the uterotrophic assay protocol by the OECD has been completed and the initial first phase of the inter-laboratory study has been published (*Kanno et al., 2001; OECD, 2001a*). The Hershberger assay has been standardised by the OECD and is currently in the process of being evaluated in a multi-laboratory study. The first phase has been completed and reported (*OECD, 2001a; OECD, 2001b*). These *in vivo* tests will be used in the first tier of screening chemicals for endocrine disruption (*Gray et al., 2002*).

There are many different fish based assays displaying different endpoints, these include fish partial chronic toxicity test and the fish whole life toxicity test which determines the effects of chemicals at different stages of the life cycle (*Ankley et al., 1998*). The most commonly measured *in-vivo* fish responses to estrogenic compounds are:

- Developmental abnormalities of the gonads (*Jobling et al., 1996*)
- Up-regulation and expression of vitellogenin (VTG) (*Hansen et al., 1998; Routledge et al., 1998; Sumpter et al., 1995; Purdom et al., 1994*).

Chemicals that inhibit steroidogenesis can be detected by a variety of *ex vivo* methods, briefly reviewed by (*Gray et al., 1997*). Generally, the *ex vivo* assay in fish measures the stimulation or inhibition of the laboratory test fish exposed to a chemical of interest. The fish is then castrated and the relevant enzyme activity is assayed. This is usually by radioimmunoassay. The vitellogenin assay, as mentioned briefly in section 1.2.1.a is a biomarker assay that measures the increased levels of vitellogenin in male fish due to exposure to an estrogenic compound. The level of vitellogenin is measured by radioimmunoassay. However, ELISA has now been established. The vitellogenin response is very specific for estrogens and the magnitude of the response is enormous and can be as much as 100,000 fold increase of vitellogenin in the blood. The disadvantage of the assay is that the structure of vitellogenin differs from specie to specie and standardisation and validation is

difficult, as a new assay has to be developed for each species. There is currently much work looking into finding the universal vitellogenin biomarker (*Hock et al., 1998*). Although *in vivo* assays are well established, the expenditure of time, cost and energy and ethical considerations do not lend themselves to large scale screening of chemicals.

1.3.1.b In vitro assays

Over the last fifteen years a large amount of *in vitro* tests have been developed for the assessment of endocrine disrupting compounds (*Eisenbrand et al., 2002*). *In vitro* assays are generally highly specific and sensitive as well as being cost effective, rapid and generally suited for high throughput screening of chemicals nonetheless, they usually only assay a single mechanism of action. Therefore, many different tests are needed to obtain a full picture of the problem. The most widely used *in vitro* test for the determination of endocrine disrupting potential can be seen summarized in table 1.5. These tests are mainly suitable for the classic receptor mediated effects. *In vitro* assays have been reviewed by (*Eisenbrand et al., 2002; Baker, 2001; Diel et al., 1999; Ankley et al., 1998*).

Table 1.5: *In vitro* test systems for the detection of endocrine disrupters

Assay type	Endpoint measured	Selected references
Cell proliferation assays	Ability of a substance to stimulate growth of hormone responsive cells (e.g. MCF-7, E-Screen)	(<i>Koltz et al., 1996; Soto et al., 1995; vom Saal et al., 1995</i>)
Reporter gene assays	Ability of a substance to activate transcription of a reporter gene construct in cell culture (mammalian/ yeast)	(<i>Arnold et al., 1999; Balaguer et al., 1999; Graumann et al., 1999; Koltz et al., 1996; Tran et al., 1996; Arnold et al., 1996b</i>)
Analysis of hormone sensitive gene expression	Ability of a substance to induce the mRNA expression of hormone sensitive genes in cell culture (e.g. pS2, Muc1)	(<i>Islinger et al., 1999</i>)
Receptor binding Assays	Binding affinity of a substance to a hormone receptor (e.g. ER, AR)	(<i>Vonier et al., 1996; Hock et al., 1998; Nishikawa et al., 1999; Gray et al., 1997</i>)
Other cell-based assays	e.g. release of FSH in Pituitary cells	(<i>Holmes et al., 1998</i>)

Cell proliferation assays

The E-SCREEN developed by Soto et al is one of the most widely used in vitro assays for the detection of estrogenic compounds (*Soto et al., 1995*). In these test systems estrogen-dependent human breast cancer cell lines such as MCF-7 (E-screen) or T47D are commonly used. The ability of a chemical of interest to stimulate growth in these cell lines is measured. This determines whether the chemical is estrogenic or not, alternatively the synthesis of new DNA or changes in the metabolic activity is determined by liquid scintillation counting or cellular enzyme immunoassay (*Diel et al., 1999*). The assay is simple and robust and has the advantage of giving a biological response. Although, widely used there have been concerns about the high inter laboratory variations in strains and culture conditions used (*Andersen et al., 1999*). It has also been reported that proliferation has been shown in response to non-estrogenic substances (e.g. progesterone, ethanol) and even some clones being insensitive to Estradiol.

Reporter Gene Expression assays

There are a number of gene expression assays in mammalian cells and yeast cells that have been developed for a range of steroid hormone receptors (*Baker, 2001*). The gene expression assays analysis the capability of a substance to activate the transcription of an EDC sensitive promoter. Eukaryotic cells (e.g. mammalian or yeast) are trans-infected with an expression vector that encodes the human estrogen receptor and a reporter gene vector. The reporter gene vector is composed of an estrogen sensitive promoter linked to a reporter gene. The reporter gene encodes a protein that has a metabolic activity that can be easily quantified (e.g. β -galactosidase that can turn the surrounding substrate medium from yellow to red)(*Diel et al., 1999*). The mechanism of this assay is shown in *figure 1.2*.

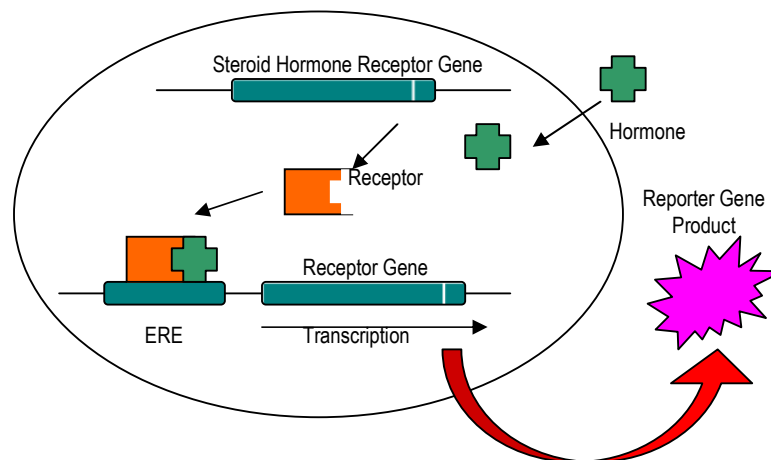


Figure 1.2: Schematic representation of a reporter gene assay. The hormone or hormone mimic has an affinity to bind to the hormone receptor synthesised by the hormone receptor gene. The hormone and hormone receptor complex are able to bond to the estrogen responsive element (ERE), which in turn illicit a transcription protein product that is able to be colourimetrically detected

The yeast cell estrogens screening assay initially established by (Routledge *et al.*, 1996) and further developed by (Arnold *et al.*, 1996b) offer many advantages for use in screening endocrine disrupting chemicals compared to mammalian cells. Yeast can be cultured easily and cheaply therefore can be used to test a high number of substances in a short period of time. Purified environmental samples can be incubated with the culture with little to no adverse affect. The yeast assay is considered to be a powerful tool in identifying substances which act via the receptor mediated pathway therefore able to determine their estrogenic potency. It can also characterise the agonistic and antagonistic properties of a substance. The YES assay is one hundred times more sensitive than radio-immunoassay for the detection of Estradiol and two times more sensitive than the E-Screen. As the assay has been developed to express a diversity of receptor (i.e. estrogen, androgen and progesterone receptors) it lends itself to being adaptable and ideal for large high though put screening of chemicals. A few limitations of the test system that have been reported, which include insensitivity of yeast cell due to impermeability of yeast cells to some test substances (i.e. dexamethasone) generating false negative results (Gray *et al.*, 1997).

Hormone sensitive gene expression analysis in cell lines

This gene expression assay measures the induction of the gene transcription following the endogenous estrogen receptor activation. It is one of the most valid procedures in characterising the estrogenicity of a substance in vitro (the YES system is in an artificial environment). The endogenous estrogen sensitive genes are imbedded in their native environment. The gene expression assay directly measures the production of the mRNA of an endogenous product stimulated by estrogens (*Diel et al., 1999; Kaplan, 1999*) these methods give semi-quantitative results and are able to give tissue specific effect information in a non artificial environment. However the analysis is time consuming and has tedious experimental protocols (*Diel et al., 2002*).

Receptor Binding assays

Steroidal hormones have a high affinity to receptors within target cell nucleuses. Receptor binding assays have therefore been developed to assess the ability of substances that can bind directly to the hormone receptor (*Baker, 2001*). The classic format of receptor binding assay is to obtain a cytotolic or nuclear extract of estrogen receptor rich tissue (e.g. mouse uterus). This extract is mixed with a combination of the test substance and a radio labelled analyte ligand (e.g. 17β Estradiol). Free unbound radio-labelled ligands are removed and measurement is determined by the percentage displacement of the radio-labelled analyte by the competing chemical. The advantage of this methodology is that tissue from a wide diversity of species can be used and the technique is widely used. However, the diversity of the level of receptor in tissue makes it difficult to obtain a standardised operating procedure (*Kaplan, 1999*).

Receptor binding assays basically use the same approach as immunoassays (please refer to section 4.1). However, they utilise the hormone receptor binding protein rather than an antibody. In recent years many non radioactive receptor binding assays have been developed (*Gray et al., 2002; Hock et al., 1998; Vonier et al., 1996*). Pan Vera (now Invitrogen Corporation Carlsbad, USA) has produced a commercially available steroidal receptor screening assay (<http://www.invitrogen.com>) based on fluorescence spectroscopy. Receptor binding assays are widely used because they are easy to perform, rapid and relatively cheap. Assays especially the ones based on the 96 well plates immunoassays (*Hock et al., 1998*), lend themselves to testing multiples of chemicals in fast succession and are suitable for large-scale screening of chemicals (*Baker, 2001*). One major criticism of these tests is that they are only suitable for hormone receptor mediated effects and hence can give false results with substances that elicit a transcription response not involving the hormone receptor (i.e. dioxins). Hormone binding receptors cannot distinguish between agonistic and antagonistic effects.

1.3.1.c Summary of effect based methods

In vitro systems are used principally for screening purposes and for generating information to validate in vivo toxicological profiles. They have the potential to be used for studying tissue and target specific effects. However, in vitro assays are generally unable to give metabolic dynamic data (i.e. pharmacokinetics: a study of what the body does to the chemical and pharmacodynamics: a study of what a chemical does to a body) that *in-vivo* assay can. Therefore, there will still be a need for animal testing for toxicological monitoring for the foreseeable future.

1.3.2 Quantitative analysis of EDC concentration

Along with the need to investigate whether a chemical is an endocrine disrupter or not in terms of screening chemicals, there is also an equal need to identify and determine concentration of EDC in environmental samples (i.e. industrial, commercial or domestic effluent). Although some of the above mentioned techniques can give qualitative measurement determination, accurate independent concentration determination is required to obtain viable monitoring data so that control measures can be implemented (*Bolz et al., 2000*). As mentioned in *section 1.1* endocrine disrupting chemicals are often low molecular weight compounds that are present at very low concentration in the environment. Ideally, these analytical techniques employed would have to have the capability of measuring samples at concentrations in the ppb-ppt (ng/L –pg/L) range and specifically identify and discriminate between different chemicals. It is also desirable that these analytical techniques are cost effective, robust and display rapid analysis time to be able to be deployed in the field or at source.

The number of analytical methodologies currently available for determination of endocrine disrupters in environmental aqueous samples are limited and generally are either biological techniques or chromatographic techniques (*Giese, 2003*). A brief general discussion of these techniques will follow.

1.3.2.a Chromatographic and Spectrometric Analysis Techniques

The most widely used chromatographic techniques used for the detection of endocrine disrupting compounds in environmental samples are gas chromatography – mass spectroscopy (GC-MS) and high-performance liquid chromatography (HPLC-MS). Of these two techniques gas chromatography is considered to be superior to HPLC as the resolution separation for compounds of similar structures is vastly better. However, GC –MS analytical technique is time consuming and labour intensive as a derivitisation step is required (*D'Ascenzo et al., 2003*). On the other hand, HPLC –MS requires no derivitisation and as a technique is quite simple to perform and can take up to ten minutes to complete a separation analysis. The lower levels of detection (LOD) for both of these techniques vary between 0.05 and 1ng/L in surface water and between 0.1 and 1ng/L for wastewater (*Lerch et al., 2003*).

In recent years additional chromatographic techniques have emerged these include liquid chromatography (LC-MS) that allows determination of steroids without derivitisation, LOD of (<0.1-5.0 ng/L) in tandem with electro-spray ionisation (ESI) and atmospheric pressure chemical ionisation detection (APCI), GC-MS-MS, LOD of (<1ng/L /ppt levels), LC-ESI-MS-MS (LOD at pg/L) and APCI-MS (LOD at µg/L or 1 ppb). LC –diode array detection (LC/DAD) method has a detection limit of 10-20 ng/L (*Jeannot et al., 2002*).

Steroidal estrogens, such as Estradiol and ethinylestradiol, have high estrogenic potencies these compounds require them to be determined at very low concentrations levels, for Estradiol and ethinylestradiol in the range of 1-10 ng/L, 0.1-1ng/L and 10-1000ng/L respectively. For xeno-estrogen like bisphenol A, which is much less potent, concentration levels required to be analysed are expected to lie in the range of 10-1000 µg/L. For chromatographic and ionisation spectrographic techniques to detect EDC at these concentration they often require a sample extraction step for pre-concentration and/or clean up step for environmental samples prior to detection as natural organic matter in concentrated extract can interfere with analysis and clog up columns. The most common extraction techniques are liquid-liquid extraction, solid phase extraction, solid phase micro extraction, pressurisation liquid extraction, soxhlet extraction and super critical fluid extraction steam distillation (*Jeannot et al., 2002*) a full and comprehensive review has been published by (*Hennion, 1999*).

1.3.3 Biochemical Based Analytical Techniques

1.3.3.a Immunoassay and immunosensor techniques

Immunoassays are well-established techniques for trace analytes in clinical, environmental and food applications; they are simple and fairly rapid analysis schemes. Combined with low cost equipment, immunoassay have developed into a strong alternative to more costly laboratory based analytical instruments such as chromatography and spectrometry (*Piebler et al., 1997*). This is because, immunoassays do not require expensive equipment for extraction or a derivative compound step and have been shown to give an excellent degree of correlation with GC-MS for analysis of steroid compounds in complex matrix like urine (*Klug et al., 1994*). Immunoassay capitalises on the high affinity binding of the antigen to the binding sites of a specific antibody. The high affinity of the antibody to the antigen can be up to 10^{-10} M that can lead to detection levels in the sub-ppb range. The potential of raising antibodies to a plethora of analytes is becoming a reality and therefore the scope of analytes that can be detected is vast. There have been many good review detailing the use and future applications for immunoassay by (*Bilitewski, 1998; Kricka, 1994; Hage, 1993*). The use of immunoassays for the detection of steroidal compounds is well established and has been in use in the clinical domain for over thirty years (*Sherry et al., 1999; Hage, 1993*). Initially radio-immunoassays were used and standard protocols were established. However, due to increase concerned over the use of radioisotopes labels alternative immunoassay schemes have been developed. It can be said that although RIA assays are still in use, EIA are rapidly replacing the original radioisotope label protocols.

Immunoassays are currently dominating the field of estrogen analysis for clinical application where estrogens levels in serum are routinely measured in patients (*Giese, 2003; IBL, 2003*). Enzymes linked immunoassays (ELISA) have been established and validated for the use of steroidal hormones in clinical laboratory. Commercially available ELISA test kits have been used and assessed for Estradiol concentration in drinking water samples on waste water samples (*Riedel-de Haën, 2003; Pan Vera, 1996*). Recently Huang and Sedlak, (2001) reported on using a commercial Estradiol ELISA kit for the determination of hormones in wastewater. Their results showed good correlation to GC-MS results with the ELISA assay displaying a higher sensitivity to low concentration than GC-MS (*Huang et al., 2001*). Immunoassay methodology and formats have been well documented elsewhere (*Baskeyfield, 2004*). These techniques (e.g. ELISA) are able to detect concentration as low as $0.01\mu\text{g/L}$, with little to no sample pre-treatment (*IBL, 2003; Mouvet et al., 1996*). However, a typical test format takes between 1-3

hours including several incubation, separation and washing steps often requiring several complex reagents and buffers (e.g. enzyme or chromagen labelling biological components and substrates). This make the immunoassay time and labour intensive, impracticable and difficult to automate for online monitoring and in-situ field testing (*Coille et al., 2002*). Ideally for an immunoassay to be applied to sample screening and in-situ monitoring, it would be highly advantageous to simplify the immunoassay procedure into a sensing system that could respond rapidly and operate continuously or pseudo-continuously. These systems that can offer this type of platform are referred to as biosensors.

1.3.4 Biosensors

During the last three decades of the twentieth century the development of novel analytical and monitoring technique has focused on three levels (1) fast, sensitive and specific devices that can be used and exploited out side a laboratory environment (e.g. bio indicators or immunoassays); (2) Advance sophisticated reliable analytical technique that can provide exact identification and quantification with high accuracy at trace levels. (i.e. GC-MS/FID/NP, HPLC-MS/UV/FD/MS etc.) And (3) Devices intended to combine the sensitive, flexibility and reliability of the above-mentioned techniques (e.g. Biosensors).

Due to the many different approaches achieved by different research groups the configurations and terms for a biosensor can vary greatly. It was Professor L.C. Clark et al in 1956 that first introduced the concept of a biosensor. The Clark oxygen electrode paved the way for many thousand of publication on biosensors and the successful multi-million commercial industry of the glucose biosensor (*Newman et al., 2002*). A generalised schematic diagram of a biosensor is shown in *Figure 1.3*.

The basic principle of a biosensor is to convert a biologically induced recognition event into a usable signal. In order to achieve this, a transducer is used to convert the (bio) chemical signal into an electronic one, which can be processed in some way, usually by a microprocessor. Recently IUPAC committee proposed a very stringent definition of a biosensor that reserves the term to be used in its modern context (*Scheller et al., 2001; Turner, 1996*) where the biological or biologically derived sensing element can be an anti-body, enzyme, receptor, cell, etc., while an appropriate transducer can either be optical, electrochemical, piezoelectric, etc (*Turner, 1996; Alvarez-Icaza et al., 1993*).

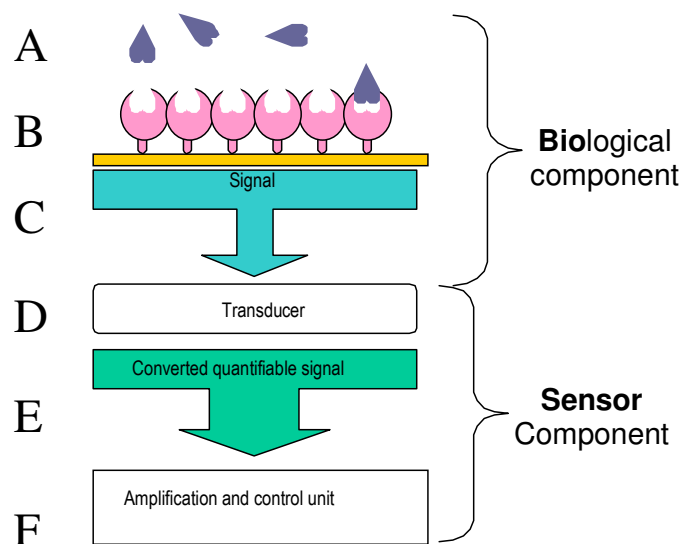


Figure 1.3: Schematic design of basic components of a biosensor. The sensing surface is often converted to a bio-specific affinity-capturing site by the immobilisation of affinity ligands to the surface (B). Once a sample containing the appropriate analyte (A) is introduced to the surface an affinity interaction creates a signal (C) that can be monitored by the sensor module and converted into a quantifiable signal (E). The amplification and read out is often a micro-processing device that is able to translate the sensors data into a manageable format (F). The separate elements can often be integrated.

IUPAC Biosensor definition:

"A biosensor is a self contained integrated device which is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element (biochemical receptor) which is in direct spatial contact with a transducer element. A biosensor should be clearly distinguished from a bio-analytical system that requires additional processing steps, such as reagent addition. Further more, a biosensor should be distinguished from a bio-probe which is either disposable after one measurement, i.e. single use, or unable to continuously monitor the analyte concentration."

(Scheller et al., 2001)

Ideally a biosensor should respond directly, selectively and continuously in the presence of target analytes when in contact with untreated collected samples. The sensor should also be able to work continuously with be able to regenerate its sensing surface to provide on-site, real or near real time accurate measurements.

In reality most biosensor only meets a proportion of the properties for example

- They use a secondary signal property ether a dye or enzymatic label for detection of primary recognition
- Sensing surface unable to be fully reversible and therefore limited to signal use (i.e. disposable).
- Compactness of sensor or miniaturisation difficult to achieve due to components and configuration to be used on-site.

Biosensors that exploit the bio-recognition of stoichiometric binding events of antibodies or biologically derived receptors are classified as affinity sensors. An appropriate transducer then detects the associated physiochemical changes. Affinity sensors are also but not inclusively known as immunosensors. Immunosensors are based on the fundamental transference of solid phase plate immunoassay onto transducer platforms (*Hock et al., 1998*). The affinity binding events are subtle and discrete and often require a sensitive transducer to translate these effects (*Mallat et al., 2001*). Contaminates found in the environment are often small in molecular size, therefore making the detection of direct binding events difficult. Thus, many devices employ the use of a secondary antibody, enzymes or chemical label or an electrochemical active substance to amplify the signal.. The sensitivity and configuration of an immunosensor depends on the transducer. There are many traducer platform in which immunoassay have been apply too (See table 1.6). Transducer applications for biosensors have been reviewed by (*Luppa et al., 2001; Sethi, 1994*).

Table 1.6: Examples of Transducers used in Biosensor Construction

Transducer	Examples
Optical	Photodiodes; Wave guide Systems; Integrated Optical Devices, Surface Plasmon Resonance, reflectometry
Mass	Quartz Crystals; Surface Acoustic Wave (SAW) Devices
Electrochemical	Clark Electrode; Mediated Electrodes; Ion-Selective Electrodes (ISEs); Field-Effect Transistor based Devices; Light Addressable Potentiometer Sensors (LAPS)
Thermal	Thermistor; Thermopile

As mentioned before with the advancement of antibody production and protein engineering techniques the scope of producing antibodies and receptors for pollutants of environmental concern is almost unlimited (*Marco et al., 1996*).

1.3.5 Immunosensors and bio-analyser systems for EDC

Immunosensors can be classified by their transducer mechanism. With the exception of the thermal transducers, examples for detecting micro-pollutants (i.e. their molecular weight <300) EDC in environmental samples can be found for all transducers and has recently been reviewed by (*Mallat et al., 2001*). The commonly used biosensors for detection of environmental micro pollutants are the optical based sensors. There are several optical transducer arrangements that have been utilised in biosensors. Many of them are evanescent field devices relying either on label-free sensing or the use of labels for their detection method (*Klotz et al., 1998*).

Coille and Reder et al (2002) describe two fluorescence optical immunoassay methods for the detection of estrogenic endocrine disrupting chemicals in water. The first is a total internal reflection fluorescent device (TIRF) developed by the University of Tübingen. The instrument, called the RIANA (River analyser) has only recently been released (i.e. 2003) as a prototype for commercial evaluation. A description and set-up of this technique has been well-documented and described elsewhere (*Coille et al., 2002; Mallat et al., 2001; Brecht et al., 1997*). The working range of the device for steroidal EDCs was 70-760ng/L. The second method described is a homogenous assay known as a energy transfer immunoassay (ETIA), again methodology and format has also been well described elsewhere (*Coille et al., 2002*). The working range for this test method was 10-850ng/L. These working ranges were achieved

without pre-concentration of the sample. Synthetic wastewater samples were tested with no pre concentration step and only sample degassing preparation. Their recovery rate was between 70-112% for TIRF and 74-110% for ETIA. Both these test system accuracy range fell well into the acceptable range according to the Association of Official Analytical Chemistry (AOAC).

Carter, Blake et al 2003 described a near real time fluorescence optical biosensor for the detection of 2,4 Dinitrophenol (2,4-DNP). The KinExA™ immunoassay instrument is by Sapidyne Instruments (Boise, USA). The methodology is well explained in the publication (Carter et al., 2003). The assay had a low detection level for 2,4DNP at 5ng/L.

Direct optical transducer methods that require no labelling are by far the more attractive platform for immuno-sensing. They not only allow simple assay procedures to be performed but also can detect and quantify the binding reaction often in real time. There are many configurations of direct optical sensors that have been used for detecting synthetic EDC in most cases for chlorinated pesticides such as surface plasmon resonance (Usami et al., 2002; Shimonmura et al., 2001), grating couplers (Mouvet et al., 1996), Mach-Zender interferometers (Drapp et al., 1997), directional couplers, reflectance interferometer fluorescence spectroscopy (RIFS) (Piehler et al., 1997) and resonant mirror (Goodard et al., 1999) have all been reported in the literature. A review of many of the label free optical immunosensors have been reviewed by (Brecht et al., 1997).

Judging by the number of papers published on the detection of endocrine hormones the preferred optical method is by far surface plasmon resonance based bio-sensing (Luppa et al., 2001). Biacore produced the first and most successful commercial laboratory based SPR biosensor platform and is one of the most developed analytical instruments for bio-molecular interactions. There are several commercially available SPR instruments; the simplicity, rapidness and direct nature of SPR with these commercial instruments have led the way to the technology to mature beyond the realm of pure research into routine bio-molecular interaction analysis. A good review of commercial SPR instruments has recently been reviewed by (Mullet et al., 2000) and informative web site detailing instrumentation, protocols and manufactures can be found at <http://home.hccnet.nl/ja.marquart/index.html>.

1.4 Surface plasmon resonance

Optical detection methods are associated with being one of the oldest and well-established techniques for sensing bio-molecular interactions. Optical transducers based on surface plasmon resonance are extremely sensitive to minute changes in refractive index that occur within 100nm of the surface of the transducer. Over the past ten years considerable advancement has been achieved in the research and development of direct optical detection methods using affinity or immunoassay based sensing. Surface plasmon resonance based optical biosensor is the most versatile and widely used transducer based optical system. As the technique is highly sensitive to the mass of the molecule associated on the sensing layer, no labelling is required which vastly simplifies the experimentation use of the instrument. The ability to monitor and measure molecular interactions as they occur in real time, provide the opportunity to determine kinetic rate constants and binding affinities as they occur.

1.4.1 Overview of surface plasmon resonance

Surface plasmon resonance is an optical quantum phenomenon that occurs when an evanescent electromagnetic field is generated at the interface of the metallic sensor surface and a non conducting dielectric medium (i.e. aqueous sample) when excited by an incident beam of light at an appropriate wavelength and at an angle just beyond the critical angle (θ_c) of total internal reflection (see figure 1.4).

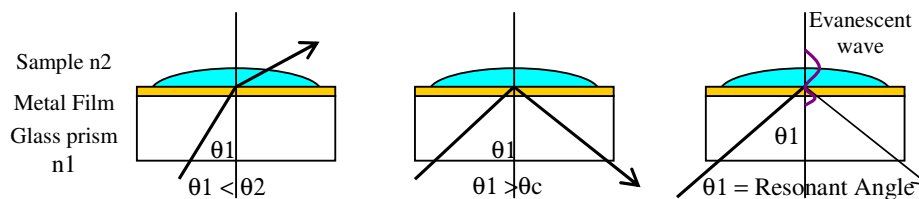


Figure 1.4: Schematic diagram of SPR principle. Light is directed through a prism of high RI into a surface layer with low RI (sample). At a particular angle Total internal reflection of the impinging light occurs. Although the light does not enter into the sample medium the intensity at the interfacial boundary is not equal to zero. The photon energy from the light is transferred to the metal electrons causing them to oscillate and produce surface bound plasmons. This produces an exponential evanescent wave that penetrate a defined distance (approx. 100 nm) into the low index medium resulting in a characterise decrease in reflected light intensity.

The evanescent wave produced by the incident beam of light penetrates a short distance into the metallic sensing surface (i.e. usually gold or silver) where the electrons are in turn excited and transformed into what is known as surface bound plasmons. The coupling of the light “photon” energy into the electrons only occurs at specific wavelengths, at this point the light energy is transferred to the “free” plasmon electrons which have a particular resonance that is different from that of the bulk of the metal film (*Liedberg et al., 1995; Löfas et al., 1991*). This coupling leads to a decrease in the amount of reflected light. If the light intensity is plotted against the angle of incident, a characteristic SPR dip can be seen (*see figure 1.5*). The angle of incidence that occurs at this point is known as the surface resonance angle (θ_{SPR}), which is dependent on the local refractive index (*Chinowsky et al., 1999*).

The refractive index is directly proportional to any material (e.g. layer thickness) bound to the metal surface or roughness. The absorption of energy by the surface plasmons (i.e. excited electrons) induces a decrease in energy of the reflected beam and thus creates a reflectance minimum. This means that within the area of the evanescent field chemical or physical interaction leads to a direct effect on the SPR angle. This causes an alteration in the position of the plasmon angle and therefore a change in the angle at which SPR occurs.

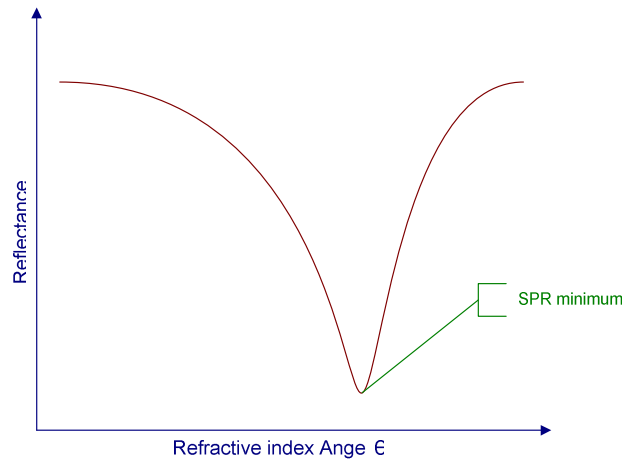


Figure 1.5: Diagram showing a SPR dip curve. The angle that the minimum reflectance occurs is known as the SPR minimum. The angle where the light energy is able to excite the surface bound electrons into excited plasmons is known as the surface plasmon coupling angle. Where this occurs no light energy is reflected back causing a reflectance minimum.

It is this angle change that is used as the basis of SPR detection devices such as Biacore® and TI Spreeta™ Systems. Because SPR is adsorbed mass sensitive it can be used to detect bio-molecular interactions at the sensing surface interface without the use of labels and subsequent reagents. Surface plasmon resonance applications has been reviewed extensively over the last ten years and more recently by (Baird *et al.*, 2001; Mullet *et al.*, 2000; Homola *et al.*, 1999; Kambhampati *et al.*, 1999).

1.4.2 Surface Plasmon Resonance and EDC Detection

Surface plasmon resonance has been utilized not only for concentration measurements of EDC in aqueous samples but also for estrogenic effects screening assays (i.e. by utilizing appropriate receptors as the biological component). Unlike traditional receptor-ligand assay systems, which rely on displacement of a radio labelled marker the SPR method allows real-time direct monitoring and additional information on kinetic affinity and dissociation between potential EDC and the responsive receptor. *Table 1.7* lists a selection of recent publication based on the detection of EDC using surface plasmon resonance.

Table 1.7: SPR technique used for EDC detection selected references.

Biological component	EDC	References
Monoclonal and Polyclonal antibodies	Dioxin (2,3,7,8,TCDD), PCB, Atrazine Estrone-3-gulcuronide, PAH	(Pearson <i>et al.</i> , 2001; Sesay <i>et al.</i> , 2001; Shimonmura <i>et al.</i> , 2001; Hock <i>et al.</i> , 1998; Tran <i>et al.</i> , 1996)
Human Estrogens receptor	Estradiol, DES, Methoxyclo Estrone, Estradiol, estriol, tamoxifen, dithylstilbestrol, bisphenol A, 4-Nonylphenol, progesterone and testosterone	(Usami <i>et al.</i> , 2002; Pearson <i>et al.</i> , 2001) (Hock <i>et al.</i> , 2002)

1.4.3 Portable/Remote Bio-sensing

Biosensors are ideal analytical devices that are well suited for integrating into portable systems for use in the field or point of care. There are many examples of miniaturisation and integrated SPR biosensors prototypes that have been designed to be compatible for use out side of the laboratory (*Huang et al., 2001; Mallat et al., 2001; Harris et al., 1999; Melendez et al., 1999; Hock et al., 1998; Brecht et al., 1997; Drapp et al., 1997; Melendez et al., 1997; Luff et al., 1996; Melendez et al., 1996; Mouvet et al., 1996; Tran et al., 1996*). Texas Instrument has developed a miniaturised sensor that lends itself to field application (*Elkind et al., 1999; Melendez et al., 1997; Melendez et al., 1996*). With the increased concern and legislation for monitoring environmental pollution the need for a rugged, simple biosensor that can be used remotely or in the field for the detection of environmental pollutants such as EDC is apparent. To date there are no real examples of SPR devices completely adaptable for use in the field. However, the University of Tübingen are currently developing an automated water analyser computer supported system (AWACSS) that will in all probability be the first automated online environmental water monitoring immunosensor. Although the detection system does not use SPR optical technique but another optical evanescent wave based technique based on TIRF, it will nevertheless help to enhance other automated optical systems to reach its level of refinement.

1.4.4 Regenerating and Reversible affinity sensors

The issue of surface regeneration of the bioactive sensing layer of a biosensor is an important one especially if the sensor is to be used continuously and or *in-situ* to its sample source. Antibody-Antigen complexes are highly stable and often require stringent buffer conditions to disrupt the binding. These reagents are often chaotropic agents, extremes ionic strength and pH, detergents, solvents and chelating agents (*Andersson et al., 1999*). These are often very similar to the common reagents used in dissociation phase in affinity chromatography procedures. The problem with these approaches is that the strong conditions can and often affects the receptor biological component reducing the binding affinity complex. There has been a concerted effort to address regeneration techniques. In a survey of 96 publications, *Andersson et al. (1999)* details a range of regeneration buffers for biosensor renewal. The author concluded that by addressing the nature of the sensing surface best solved the problem of surface regeneration, rather than the type or reagent used. Therefore, an ideal scenario would be to develop a sensor that has a biological surface that was able to reversibly bind to its affinity partner via a non-evasive regeneration step.

Kagner, Pogreb *et al.* (1999) Published a paper demonstrating a buffer-less regenerating surface plasmon resonance biosensor for 2,4, DNP. By immobilizing a photochromic dye (dinitrospiropyran) as the antigen partner on the SPR gold surface they found that anti-DNP would bind to the surface. Dinitrospiropyran is a photoisomerizable compound that exists in two very different forms when illuminated at different wavelength. After the completion of a sensing event the surface was illuminated at an appropriate wavelength, the bound anti body had little to no affinity to the isomerised antigen from that the antibody was able to be washed away with the buffer flow to form a regenerated surface (*Willner et al., 2001; Kagner et al., 1999*). This paper demonstrated true non-evasive regeneration of an immunoassay on a bio sensing system.

1.5 Conclusion

In conclusion, the increase in incidence of hormone dependent diseases (e.g. breast cancer, testicular cancer and prostate cancer) has intensified public concern of possible exposure to endocrine disrupting chemicals in our environment especially in the aquatic environment. It has been observed that EDCs in particularly estrogenic exogenous hormones and hormone mimics are present in minute quantities in waste waters and surface water and have been seen to have feminising effects on aquatic wildlife. The possible environmental burden they pose to humans has created a need to develop new analytical devices and techniques that are able to monitor these pollutants in environmental samples and at source. To date several techniques exist for measuring estrogenic compounds for clinical and pharmacological purposes. Monitoring and measuring estrogenic compounds in environmental samples is a relatively a new area and one that currently needs to be evaluated, validated and standardised. The development of new analytical techniques especially for the detection EDC at waste water treatment works and sewage treatment works would be advantageous as it would enable them characterise, monitor and reduce the level of these compounds they release in their effluents.

1.6 Aims and Objectives

The broad aim of this project was to develop a portable, field based surface plasmon resonance immunoassay device. The device was to be used primarily to detect estrogenic endocrine disrupting chemicals in wastewater and surface water samples. The requisite for the portable surface plasmon resonance sensor was so that the device can be implemented near to or at a wastewater treatment plant.

The approach taken in this research work was therefore, to design a portable device around the miniaturised Texas Instrument surface plasmon resonance sensor (Spreeta™). The prevalent characteristics of this sensing system and assay methodology was to achieve a simple immobilisation protocol for the biological sensitive surface, employ an inhibition based immunoassay for the detection of endocrine disrupting chemicals and be able to regenerate the sensor for further use. Sensor regeneration is an important area of this research and a study of sensor surface regeneration for remote immunoassay detection was investigated. To this aim a number of key areas and objectives were to be examined:

1.6.1 Construction and Characterisation of a SPR Analyser (Chapter 2)

The main aim of the work reported in this chapter was to construct and characterise a portable SPR sensor device for the detection of a model steroidal estrogen (Estrone-3-Gulcuronide). A range of immobilisation techniques and regeneration buffers was explored to develop an immunoassay protocol that would be used with the sensing platform.

1.6.2 Refinement, Development and Optimisation of an EDC Immuno Analyser (Chapter 3)

The aim of the work reported in this chapter was to refine and optimise the immunoassay protocol for the detection of estrogens. Optimisation and validation of the potable analyser was to be determined by developing an ELISA plate assay for Estradiol. The system was then to be tested using spiked samples in synthetic and real wastewater. It was intended in this chapter to determine the sensitivity and working range of the portable device

compared to the developed ELISA plate assay, a commercial ELISA plate immunoassay and the Biacore™ platform.

1.6.3 Photo-modulation of Bio-active Proteins for sensor regeneration applications (chapter 4)

Within this chapter the photo-modulation of bioactive proteins conjugated to a spiropyran dye was examined. To this objective, the dye was synthesised and characterised before being conjugated on to the protein. Two bioactive protein systems (i.e. enzyme and antibody) were investigated. It was anticipated that photo-modulation of these proteins could be achieved by attaching the dye to the proteins and hence change their normal active function. This being so, the dye modified proteins were then applied to immunoassay systems for the application of a remote regeneration format.

2 Construction and characterisation of a SPR analyser

Chapter 2

Construction and characterisation of a SPR analyser

2.1 Introduction

Environmental monitoring for the detection of pollutants is an increasingly important issue for regulatory and legislation agencies, regulated industries and the general public. This also holds particularly true for compounds like EDCs that pose a potential health risk to humans and wildlife in the natural environment. The current high cost and slow turnaround period associated with the measurement of regulated pollutants, clearly indicates the need for environmental screening and monitoring methods that are fast, portable and cost effective. Biosensors, especially those based on optical sensing (e.g. SPR) are ideally suited for this specific application niche. Increased legislation and quality assurance for environmental control has meant that *in-situ* analysis for medical, environmental, food safety, bioprocesses and for military use (i.e. detection of biological warfare agents and explosives) have increased the demand in this area for an adaptable, inexpensive, robust and field deployable analyser.

2.1.1 Development of the miniaturised sensor

There are a number of commercially available SPR instruments designed for real-time affinity-binding studies (e.g. Biacore®, Biacore AB Uppsala, Sweden; Iasy®, Affinity Sensors, Cambridge, UK; IBIS iSPR, IBIS Technologies BV, BE Hengelo, Netherlands; Plamonic, Jandratek GmbH, Ellenfels, Germany; etc). These instruments vary in sensitivity, function and cost. Recent reviews compare the key features of these commercial instruments and gives a general background on SPR sensing (*Leonard et al., 2003; Baird et al., 2001; Homola et al., 1999*). It would be true to say that bio-sensing of bio-molecular interaction using SPR technique has now become an established routine procedure with a vast amount of publications detailing methodologies and procedures for analyte detection (*Rogers, 2000*). However, many of these commercial instruments are bench top equipment not suited for field applications.

Texas Instruments launched the first miniature SPR sensor in 1996. In development since 1995, the sensor then called TISPR-1 was and still is the first integrated optical sensor that has reached commercialisation. No bigger than a matchbox, it was initially hand made and was available as a single evaluation module sensor with the gold surface pre-evaporated on it. In 1999, Texas Instrument introduced to the market a fully manufactured sensor in which they distributed in units of 50 and renamed to Spreeta™ SPR sensor. This new sensor has made way for the real possibility of being adapted into a small portable analyser with considerable advantages, as it is robust with no moving parts and inexpensive. Texas instruments will launch a new multi-channel Spreeta™ sensor (i.e. 3 or 5 channels) in 2004 [John Wisehart, Normadic, Ohio, USA; personnel communication].

2.1.2 Previous Work Using the TI-SPR and Spreeta™ sensor

The Texas miniature SPR sensor has been in development for over eight years. Over the course of this research project a total of 15 papers using the sensor as a biosensor have been published. There are ten papers that describe experiments performed on the sensor and three papers that have reviewed the technology. Kukanskis et al. used the sensor for the detection of DNA hybridisations and although they were unable to quantify their end product they presented useful data and information of the sensors limitation (*Kukanskis et al., 1999*). This work was instrumental in the improvements of the system in terms of baseline noise stability.

Four recent papers have used the sensor and presented quantitative detection of analytes of environmental interest using an antibody-antigen immunoassay. The first, describes the sensor being used for food allergen detection and demonstrated that peanut allergen could be directly measured down to a detection limit of $0.7\mu\text{g/ml}$ (Mohammed *et al.*, 2001). The second paper reported on the detection of Staphylococcus aureus enterotoxin B (SEB), as a model system for the detection of biological warfare agents. The results were performed on a dual channel sensor and was able to directly detect the toxin down to femtomolar (fM) levels in buffer, milk and urine samples (Naimushin *et al.*, 2002). In the third paper the Spreeta™ sensor is compared to a miniature quartz crystals microbalance sensor for the detection of Escherichia coli heat-labile enterotoxin. By using a direct immunoassay detection method they were able to measure *E.coli* enterotoxin with a lower detection limit of 70pmol of the toxin. The authors conclude that in their opinion the sensor was well suited to remote sensing for flow through systems provided that suitable enclosures were provided (Mohammed *et al.*, 2001; Spangler *et al.*, 2001). The last paper described the detection of trinitrotoluene (TNT) in environmental soil samples. Unlike the other publication it describes an inhibition immunoassay where the antibody is pre-incubated with the sample. In a blind study using 180 spiked soil samples in three different soil matrixes it was reported that the assay sensitivity had a lower detection limit of 1ppm TNT with no false negatives (Strong *et al.*, 1999).

In all of these papers published, surface immobilisation of the bio-active sensing layer and sensor surface regeneration was an important issue as assay development and procedures are restricted by the unique design of the sensor.

2.2 Aims and Objectives

The aim of the work presented in this chapter was to install and characterise the Texas Instrument miniature SPR sensor with the intention of converting the sensor into a portable field analyser. The objective of the study was to be able to use the portable SPR analyser for the detection of endocrine disrupting chemicals in waste and surface waters. A monoclonal antibody against Estrone-3-Glucuronide (E₁3G) was to be used as a model immunoassay antibody system for the detection of estrogenic compounds and hormone mimicking compounds. To this end the development of an effective fluid handling system for applying samples to the sensor and the improvement of the systems signal and noise ratio to enhance the measurement sensitivity was paramount. The initial development of an *in-situ* detection assay to be used on the analyser was sort after with a study of different methods for preparing the biological sensing surface and an appropriate regeneration protocol was also to be established. The regeneration of the sensors sensing surface was considered an important feature in the study as the portable analyser was primarily aimed to be used in the field.

2.3 Equipment

2.3.1 Surface Plasmon Resonance instruments

The Texas Instrument Spreeta™ SPR sensor used in this research was purchased from Normadic Ltd. (*Stillwater, Oklahoma, USA*). The Biacore system used was the Biacore® 3000, Biacore® installed all software. The sensing chips used in this study were the J1 plain gold chips. All consumable materials used were purchased directly from Biacore® (*Stevenage, Herts., UK*).

2.3.2 Static contact-angle measurement

Contact angle measurements (Sessile drop method) were used to characterise the hydrophobicity / hydrophilicity of the sensing surfaces produced under various experiment conditions. Images of individual drops were captured using a CCD camera (*Spectral Source, California, USA*) with a F1.4 lens. The CCD camera is powered and controlled via a PC interface board and the software allowed image acquisition and processing to occur. To calculate the relevant drop dimensions in pixels the images were viewed in Adobe Photoshop™ LE. Contact angles were taken from 1µl drops of water.

The measurements were performed in a transparent environmental chamber; this was in order to maintain a humid saturated atmosphere to prevent drop evaporation. The 1µl drop of water was delivered on to the surface using a Hamilton syringe. The needle was held in place by a laboratory stand and the drop was held on the needle. The prepared surface was raised until the drop was transferred to the sample surface and then lowered; the resultant water drop image was taken. A typical contact angle image is show in *Figure 2.1*. For each surface, six drops were prepared and measured to give a mean contact angle and standard deviations were calculated.

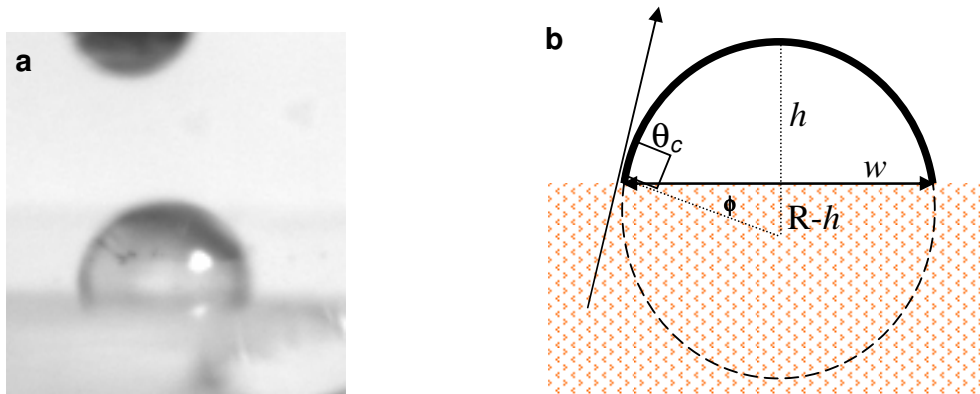


Figure 2.1(a+b): An example and schematic representation of a contact angle measurement: a) CCD image showing a typical drop of water (1 μ l) deposited on to a gold surface. b) Schematic representation showing parameters used to calculate the contact angle. Drop dimensions were calculated using image analysis software. Calculations were performed according to the mathematical method as described in the main text.

$$\text{Equation (1):} \quad R = \frac{(0.25w^2) + h^2}{2h}$$

$$\text{Equation (2):} \quad \theta_c = 90 - \left(\tan^{-1} \left(\frac{R-h}{0.5w} \right) \right)$$

$$\text{Equation (3):} \quad \phi = \tan^{-1} \left(\frac{w^2/3h - h}{w} \right)$$

The formula in equation (1) was used to obtain drop radius, R . Equation (2) and (3) gives the calculations required for obtaining the contact angle θ_c and ϕ angle for the measured drop. Where h is the drop height and w is width of drop at the contact area with the surface (Gillespie, 2001).

2.3.3 Atomic Force Microscopy

2.3.3.a General Theory

Unlike traditional microscopes, scanning-probe systems like the Atomic Force microscope, does not use lenses. The AFM utilises a sharp probe that moves over the surface of a sample in a raster scan. Therefore, the size of the probe rather than diffraction limits their resolution (*Albrecht et al., 1991*). AFM operates by measuring attractive or repulsive forces between the tip and the sample (*Binnig et al., 1986*). The probe is a tip on the end of a cantilever, which bends in response to the force between the tip and the sample. The AFM utilizes an optical lever technique.

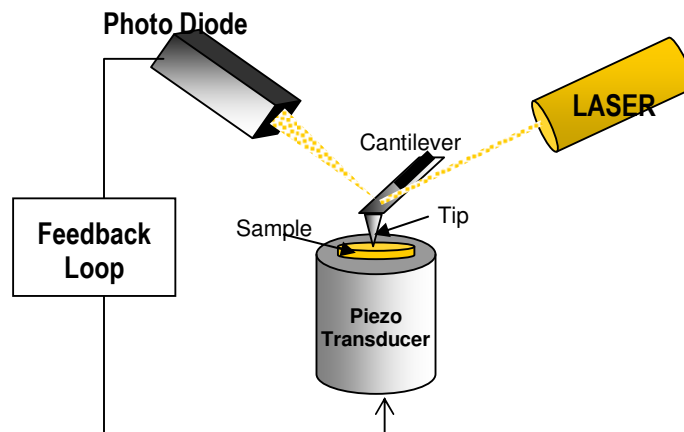


Figure 2.2: Schematic representation of the atomic force microscope. The AFM instrument consists of a cantilever tip, that depending on the method of scanning is either dragged (contact), tapped (tapping) or simply skimmed over the surface (non contact, depending on sample surface and tip attraction forces to each other.). A laser beam illuminates the cantilever and is then reflected back onto a split photodiode that is able to discriminate the vertical flex position due to the sample surface roughness. The feed back loop maintains a pre-defined set point of the tip in relation to the scanned surface.

The diagram in *figure 2.2* illustrates how this works; as the cantilever flexes, the light from the laser is reflected onto a split photo-diode. By measuring the difference in signal between the two different split sides of the diode, changes in the bending of the cantilever can be measured. Since the cantilever obeys Hooke's Law for small displacements, the interaction force between the tip and the sample can be found. The movement of the tip or sample is performed by

an extremely precise positioning device made from piezo-electric ceramics, most often in the form of a tube scanner. The scanner is capable of sub-angstrom resolution in x -, y - and z -directions. The z -axis is conventionally perpendicular to the tip-sample interaction (i.e. in a vertical plane to the sample substrate).

The probe image contrast can be achieved in many ways. The three main classes of probe interaction are contact mode, tapping mode and non-contact mode. Contact mode is the most common method of operation. When scanning in contact mode the tip and sample remain in close contact. One of the drawbacks of allowing the probe to remain in contact with the sample is that large lateral forces are placed on the sample as the tip is "dragged" over the sample. In tapping mode the probe is periodically in contact with the surface. This method and non-contact mode is usually used when scanning more surface sensitive surfaces where applied lateral forces are kept to a minimum. In non-contact mode, the AFM derives topographic images from attractive force measurements between the tip and sample where the tip does not touch the sample at all.

2.3.3.b AFM equipment and settings

The AFM scans were performed using the PICOSPM™ atomic force microscope (M-Scanner, *Molecular Imaging, Phoenix, USA*). The microscope allows simultaneous imaging of the surface, topography, cantilever deflection, and friction (lateral force). Surfaces were scanned using contact mode, silicon nitride probes (model CONT-16: *Nanosensor GMBH, Wetzlar-Blankenfeld, Germany*), with a nominal spring constant C of 0.05N.M^{-1} . Scan rates of 1 Hz with a resolution of (10,000) scan lines per image at scan dimensions of $10 \times 10\mu\text{m}$.

2.3.4 The Spreeta™ sensor concept and design

The Texas Instrument TSPR1XXX is a family of integrated optical analytical devices. They can operate in two modes (1) They can be used as a refractometer able to measure the refractive index (RI) of liquids in contact with its surface, or (2) as a biosensor: when the integral sensing surface is modified by a bioactive surface allowing biological / chemical binding events to occur at the sensing surface.

The sensor incorporates all the electro- optical components required for an SPR instrument mounted and wired bonded onto a miniature (400mm^2) platform using normal semiconductor-based opto-electronic manufacturing techniques (*Melendez et al., 1999*). These assembled components are encapsulated in a clear, optical epoxy resin (RI 1.52) using a cast moulding processes. The epoxy encapsulation is moulded to form a Kretschmann geometry prism to facilitate the excitation and detection of surface plasmon resonance. The resin moulding also provides all the necessary optical surfaces where no optical alignment is necessary and complete protection of internal electronic components. The sidewall of the old hand made TSPR-1 sensor is coated with an opaque film to block out external light. The Spreeta™ sensor is encased in black plastic (*see figure 2.3*).

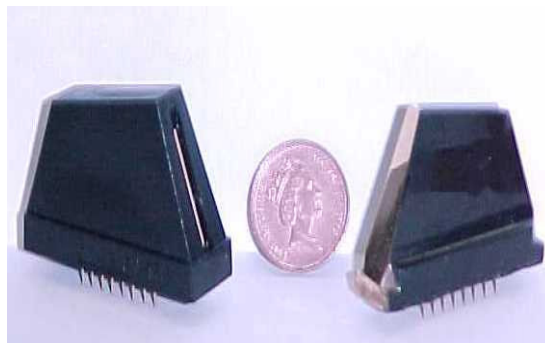


Figure 2.3: The Texas instrument miniaturised SPR sensors. The sensor on the left shows the second-generation Spreeta™ SPR sensor, it differs from the sensor on the right, which is the original hand made TISPR-1 sensor in that it is manufactured with a recessed gold sensing surface. Each sensor weighs approx. 10g.

The sensor contains a near-infra red light-emitting diode (ALGaAa LED) with a wavelength of 840nm , the emitted light is collimated and polarised with a p -polariser, and the LED is housed in a light absorbing material which includes silicon light to voltage chip that monitors the LED intensity variations. The top of the housing has an aperture that allows light to enter the system. A polariser is attached to the opening to allow only transverse magnetic radiation into the system as trans-magnetic radiation (TM) can excite surface plasmon. The light emitted is incident on the gold sensing surface with a range of light incident angles (i.e. creating a fan of angles). As the geometry of the prism is in a Kretschmann configuration the incident beam creates surface plasmons on the dielectric surface (see figure 2.4). The emitted light beam is reflected off the gold surface onto the mirrored surface and is then directed onto the photodiode array. Each pixel detects the light reflected for a particular angle of incidence. All these components including an internal thermistor are seated on a printed circuit board, which has 16 contact pins. The system electronics interface occurs through these contact pins laid out in two rows of 8 pins. These contact pins provide an output from the array to a signal processor, which in turn quantifies the resonance conditions and sensor temperature to give the interrogated liquid sample's index of refraction. The active sensing region is a strip approximately 1.5mm long by 0.1mm wide on the face of the sensor.

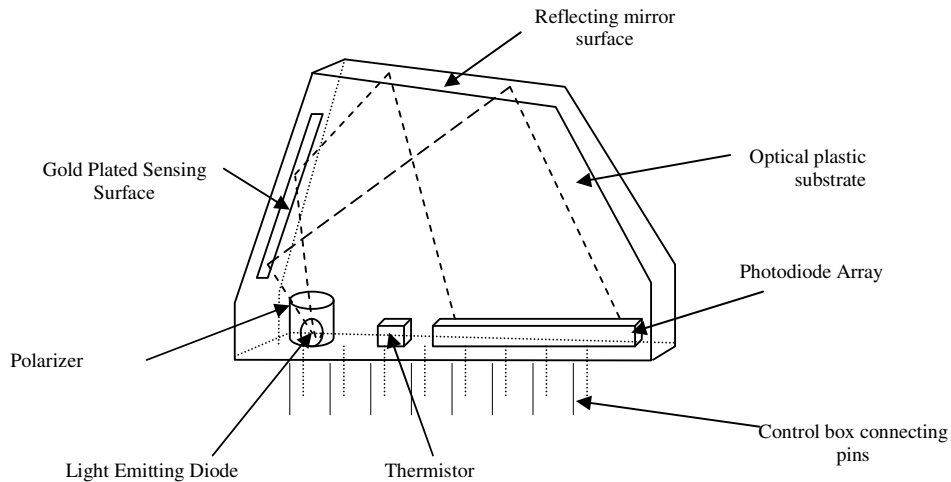


Figure 2.4: Schematic of internal structures of the Spreeta™ sensor: The sensor incorporates an LED, p -Polariser, a thermistor and silicon photodiode array. All of these components are mounted on a printed circuit board and are encapsulated in clear optical epoxy resin. The gold sensing surface has been thermally vacuum evaporate on the surface at a thickness of 50nm over a thin layer of chromium to aid adhesion to the surface. The contact pin allows for connection to the control box for sensor operation.

The Spreeta™ sensor unlike most SPR systems available has the gold sensing surface pre-evaporated on the sensor chip therefore, making it an integral part

of the system. This means, when wanting to reuse the sensor it is vital that the gold sensing surface is clean and intact. Alternatively, the gold surface could be removed and replaced by evaporating a new gold sensing surface. With the TISPR-1 sensor a gold slide kit could be used by index matching pre-evaporated gold glass cover slides to the sensor surface. The second-generation Spreeta™ sensor used in this report had a reset gold surface. This meant that replacement of the gold surface was virtually impossible. Therefore, the issue of gold surface regeneration was paramount for the design, construction and application of an immuno-assay based field deployable analyser.

The sensor is provided with dedicated software, a flow cell, connection leads and an 8-bit analogue to digital micro-control interface box. The Spreeta™ flow cell was secured on the sensor with a G clamp (*please refer to figure 2.5*)



Figure 2.5: *Photographic illustration of the original Spreeta™ G-clamp flow cell. The flow cell is connected to the SPR sensor by using a G-Clamp. The sensor is then connected to the control box via the connection lead. Fluid is introduced to the sensor surface via tubing of 0.25mm diameter.*

2.3.4.a The constructed portable sensor analyser

The experiments carried out in this chapter were using the Spreeta™ evaluation module kit manufactured by Texas Instruments Inc. (TI), distributed by Nomadics Inc (Stillwater, Oklahoma, U.S.A.). The commercially available evaluation package consists of 50 miniature SPR sensors, and associated peripherals. The Spreeta™ sensor was integrated into a self-contained analyser (*please refer to results section 2.6.1*). The analyser comprised of a steel housing (RS Components, Corby, UK) containing the Spreeta™ SPR device, flow cell, control electronics, manual sample loop injection valve and liquid switching valve and Teflon tubing: 1/16" with an inner diameter of 0.25mm (Ominfit, Cambridge, UK) and reagents reservoir bottles (BDH, Poole, UK).

Reagents and buffers were initially pulled through the flow cell using an external peristaltic pump Miniplus 3, Gilson (*Anachem Ltd, Luton, U.K.*).

2.3.4.b Spreeta™ Software Set up

The Spreeta™ SPR_MINI.EXE software is a window application written in Visual basic. Several software parameters were set for the experiment presented to ensure compact data files and low system noise. These settings were:

- The number of automated measurements was set to 20 therefore the SPR data output over 20 recorded data events value (angle and pixel number) was averaged to make one point.
- The minimal monitoring interval time required to drive the sensor and analyse the result was set to 0.25 (i.e. $20 \times 0.25 = 5$ sec. per point).
- Every other data point event was recorded (i.e. 10s between each saved data value).
- The SPR curve was smoothed using the 13-point method option and temperature compensation was set.
- Analysis methods were each assessed for their ability to monitor the SPR minimum shift and hence refractive index changes (See section 2.7.1.1).

2.3.4.c Spreeta™ sensor data collection and data analysis

The automatically recorded data according to the parameters set above were viewed from the data table and transferred directly into Microsoft Excel 97 spreadsheet. The time verse refractive index or angle sensor-gram scans obtained were analysed manually to obtain changes in angle over time and relevant information on signal to noise data.

2.4 Materials

All chemicals and bio-chemicals were of analytical grade and were obtained from Sigma-Aldrich (*Pool, Dorset, UK*) unless otherwise stated.

2.4.1 Chemicals

Albumin (chicken egg) [code: A5503] also known as Ovalbumin (OVA), Bovine serum albumin (BSA)[code: A7030], Phosphate buffered saline tablets (PBS) pH 7.4 [code: P4417], Phosphate buffered saline, pH 7.4, with Tween 20 (PBST) [code: P3563], 2-[N-morpholino] ethanesulfonic acid (MES) Buffer pH 6.7 [code: M0164], Triton X-100 [T9284], 1-Ethyl-3-(3-Dimethylaminopropyl) carbodiimide (EDAC) [code: E7750], N-hydroxysuccinimide (NHS) [code:H-7377], Sucrose (ultra) [code: S-7903], Protein Assay kit [code: 690-A], and 3-aminopropyltrimethoxysilane (APTS) [code: A3648, Glycine [code: G6761], hydrochloric acid [code: H1758], Triton X –100 [code: T 9284], Tween 20 [code: P1379], Sodium dodecyl sulphate (SDS) [code: L 4509], and Sucrose [code: S7903], were obtained from Sigma-Aldrich (*Pool, Dorset, UK*). Analar grade water [102927G], Sodium Hydroxide pearls (NaOH) [code: 307314P], Hydrogen chloride (HCL) [code: 101254 H, 99.5%] was obtained from BDH laboratory supplies (*Poole, Dorset, UK*), Persil biological liquid (*Lever brothers, Port Sunlight,U.K*).

2.4.2 Antibodies, conjugates and analytes

Monoclonal antibodies against Estrone-3-Gulcuronide (E₁3G: clone 4155), human gonadotrophin (HGC: clone 3299) and ovalbumin-E₁3G conjugate were a kind gift provided by Unilever Research Plc (*Bedfordshire, U.K.*). Antibodies were stored at concentrations of 100µg/ml and frozen in aliquots of 200µl and 100µl. Protein conjugate aliquots were stored in the fridge at 4°C. Calibration samples of Estrone 3 –(β-D-Gulcuronide) sodium salt [E1752] E₁3G (*Sigma, Poole, UK*) was prepared by dissolving 1 mg of E₁3G in 1ml of dimethylformamide (DMF) and then making a stock solution at 1µg/ml with phosphate buffer saline pH 7.4 with 0.5% Tween 20 (PBST). Calibration standards were prepared in 2 ml brown glass bottle at 4°C until used.

2.4.3 Buffers and reagents

The following regeneration buffers were used to ascertain their effectiveness in the regeneration of the gold sensing surface of the sensor:

Glycine (10 mM), SDS (1%) (wt/vol.) and HCl to give pH 2.7

Sodium hydroxide (0.12N) and Triton X-100 (0.1%) (wt/vol.), pH 13

Persil Biological (liquid form) (1%) (vol./vol.)

Before use, the buffer was degassed by ultra sonicfication, equilibrated to room temperature and filtered with a 0.45 μm syringe filter (*Fisher Scientific Ltd, Loughborough, Leicestershire, UK*).

2.4.4 Other Materials

Microscope slides [code: 406/0180/02], Glass cover slips [code: 101254 H] obtained from Merck Chemicals Ltd. (*Poole, Dorset, UK*).

2.5 Methods and Procedures

2.5.1 Measuring refractive index using the Spreeta™ sensor

The Spreeta™ dedicated software package (version 4.2) was used during the course of the work described in this thesis. The software enables the user to operate the sensor and make adjustments to the sensor settings. The SPR dip required for determining the refractive index is resolved by the measurement of reflected light intensity on individual pixels on the photodiode array.

Before a new sensor is used for measurements it needs to be first calibrated in air. Commanding the air initialisation button carries this out. Air initialisation takes the background reference values of the reflected light with and without the LED on. Air initialisation varies the LED intensity and measures the light signal; this allows the photodiode integration times to be optimised by finding the maximum light signal without saturating the output (this can also be checked and set manually by looking at the raw data via the view button of the software).

The refractive index in air is very low and as the Spreeta™ sensor has a dynamic refractive index range of 1.320 –1.368 (refractive index units: RIU at 840nm), a SPR response in air is impossible and a flat baseline is seen (i.e. this should be approx.: 1.00 RIU as this corresponds to the value of the refractive index of air. The refractive index of water is 1.33 RIU. Therefore, by calibrating the sensor against the know refractive index of water, enables further refractive index values to be calculated. This is performed by filling the flow cell with water and executing the “Calibrate with water” button which automatically sets the refractive index of 1.33 for water to the pixel number corresponding to the SPR dip obtained (See figure 2.6).

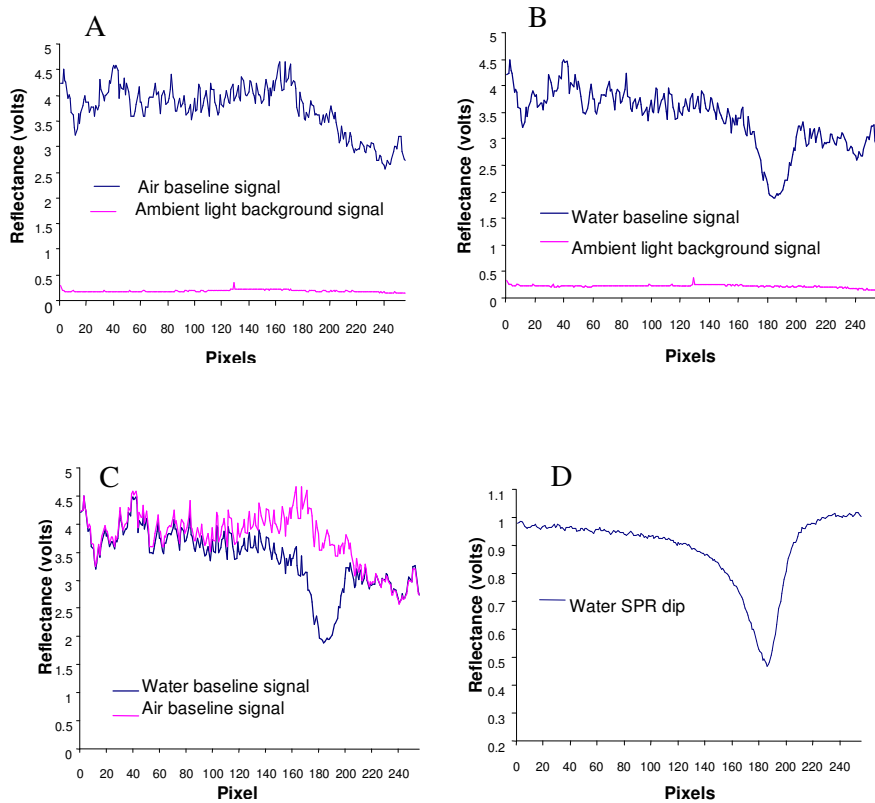


Figure 2.6: Spreeta™ resolved SPR dip: (A) shows the baseline SPR signal of the sensor in air and the ambient background light signal (this is taken with the LED turned off). (B) Shows the baseline signal for the sample and again a background baseline is taken (i.e. LED is off). The water and air reference are subtracted from each other (C) and the SPR dip curve is realised (D). (Measurements were performed on TI-SPR1 Sensor).

2.5.2 Analysis Method Parameters

The Spreeta™ associated software has seven different analysis methods available in which the SPR dip sensor data analysis can be interpreted and monitored to give a typical SPR sensor-gram or refractive index co-ordinate information. Once the SPR curve has been plotted, the position of the resonance must be determined in order to calculate the samples refractive index. Under ideal circumstances the position of the SPR curve minimum determines the refractive index. The software analysis methods allows flexibility in regards to data analysis as some of the methods determines the dip position and others extrapolate the position of the dip and follows the position of the SPR curve should it shift with time. Five of these methods were investigated to determine the most appropriate method to be used for further work as each method has its strengths and weaknesses.

Method 1: First moment of the SPR curve above or below a set baseline.

This method requires the baseline to be set where the SPR curve gradient is at its greatest (i.e. 1st derivative maximum); this is nominally around 80% of the measured reflectance. The direction of the moment is calculated above or below the baseline (usually below). A vertical line bisecting the horizontal baseline is determined to be the position of the first moment (*see figure 2.7*)

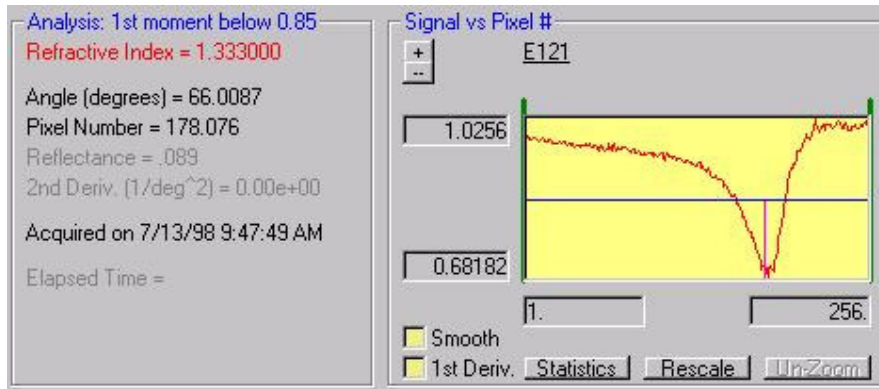


Figure 2.7: Software picture showing the First moment Analysis method. The blue horizontal line is set nominally where the SPR gradient is at its greatest. A vertical line bisecting the SPR dip determines the lowest point of the SPR minimum.

Method 2: The point of specific Reflectance /signal

This method observes the pixel position where a particular reflectivity occurs nominally at 80% reflectance. It does this by searching for an approximate

point where the reflectance occurs. Then it performs a 4th order polynomial fit about the pixel point. Using the fitting result the method interpolates the point where reflectivity occurs (*please refer to figure 2.8*).

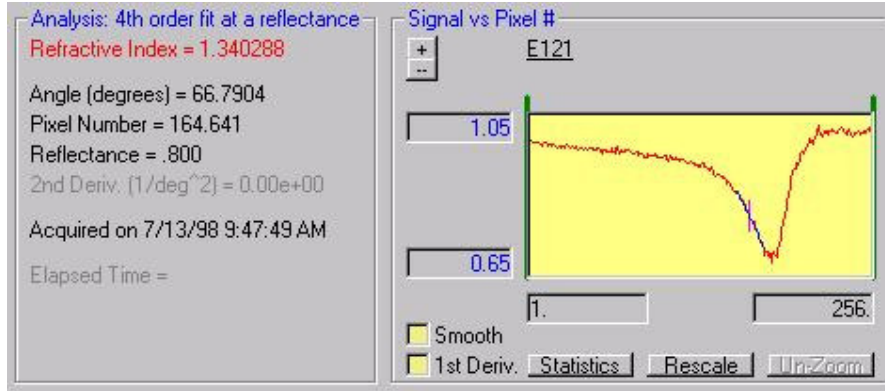


Figure 2.8: Software picture showing 4th Order specific reflectance/signal analysis method. This method approximates the position of a specific reflectance (nominally at 80%) and then performs a 4th order fit around the reflectance pint to find the position.

This method follows the position of a specific reflectance point with time rather than the actual SPR dip minimum.

Method 3; Polynomial fit of the resonance minimum

This method finds the rough minimum point of the SPR dip and then performs a 4th order polynomial fit about the rough minimum. It then uses the fitting results to find a fine minimum point to perform another 4th order polynomial fit about the fine minimum where the SPR minimum position is determined (*see figure 2.9*).

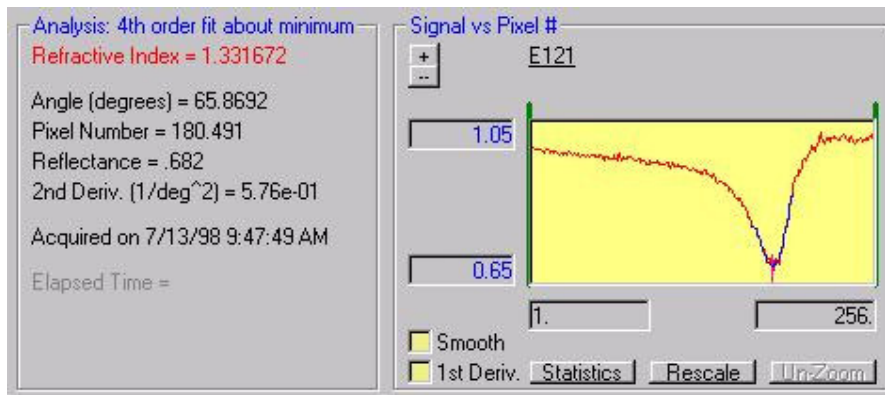


Figure 2.9 Software picture showing the Polynomial fit of the Resonance Minimum Analysis method. A 4th order fit is performed to find an approximate position of the SPR dip minimum. Another 4th order polynomial fit is performed at this value to determine the minimum position.

This method like the First moment analysis method follows the SPR reflectance minimum with time.

Method 4: Zero crossing of the derivative

This method initially finds a rough minimum point of the SPR dip and performs a 4th order polynomial fit about the rough minimum. A linear fit of the derivative about the rough minimum is performed and the zero crossing point from this fitted data is used as the fine minimum. The method performs another linear fit of the derivative about this fine minimum. The fitted data is then used to find a new zero crossing point in which it is taken to be the SPR minimum (see figure 2.10).

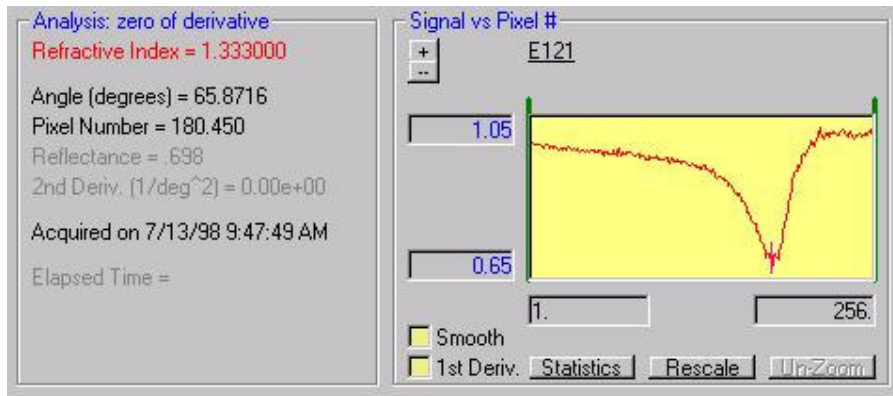


Figure 2.10 Software picture showing the Zero Crossing Analysis method. An approximate position of the SPR minimum is found by performing a 4th order polynomial fit about the minimum. A linear fit is performed on this point and the zero-crossing point is used to find the fine minimum.

This method like the first moment analysis and 4th order polynomial fit about the SPR minimum follows the SPR dip minimum with time.

Method 5: Dot product of data with derivative

This method calculates the changes in Refractive index verses time. Therefore a preset Refractive index value is required. In this method the SPR curve is multiplied (in a dot product fashion) with the derivative of the SPR curve at time = zero. The change in this product with time is directly related to changes in the refractive index.

2.5.2.a Refractive index measurements using sucrose standards

To validate the use of the sensor as a refractometer and to compare the sensitivity of the sensor to the Biacore® 3000, different sucrose solutions in succession were applied to the sensor surface. The flow rate and duration applied was 10µl/min for 5 minutes for both SPR equipments. The sucrose refractive index standards used for refractive index calibration were 10, 20, 30 and 40 % (wt/vol.). For bulk refractive index comparison analysis between both the Spreeta™ and Biacore®3000 equipment, sucrose solution of 0.1, 0.25, 0.5, 1, 2.5 and 15 (%wt/vol.) were used.

2.5.2.b Flow rates and refractive index stability

Flow velocity of a fluid in contact with the sensing surface can affect the bulk refractive index measured. To determine the influence that flow rates has on the stability of the refractive index over a period of time and to find a suitable flow rate that gives low signal to noise levels, a refractive index versus time trace sensor-gram of PBS (at room temperature and degassed) was passed over the surface for 15 minutes at five different flow rates. The short-term noise envelope and standard deviation was compared. This was carried out with PBS passed over a clean sensor surface and then with PBS passed over a surface where BSA was physical adsorbed on the sensing surface. The BSA surface was prepared by passing a solution of 50µg/ml of protein over a clean sensor surface for 10 min at 10µl/min and then rinsing for five minutes with PBS (*please refer to section 2.7.2*).

2.5.3 Surface preparation and sensor surface modification methods

For the Spreeta™ sensor to be converted into a biosensor the gold sensing surface needs to be modified by attaching a biological active surface on to it. This can be done by physical adsorption, chemisorption and covalent attachment (*Hermanson et al., 1992*). The following sections describe methods and procedures carried out in preparation and modification of the gold sensing surface.

2.5.3.a Spreeta™ Sensor surface cleaning

The gold sensing surface was washed and dried using an isopropyl alcohol (IPA) (90%) and an airbrush, this was used to dissolve and remove any unwanted protein and grease marks from the surface. The sensors were then placed into a beaker of HCl (1M) and ultra sonicated for 10 minutes and rinsed with Analar or RO water. Before use the sensor was thoroughly dried with nitrogen and fitted into the flow cell for subsequent analytical measurements or immobilisation of the biological active layer.

2.5.3.b Glass Slide cleaning

Glass slides were cut into 1cm² squares and were ultra sonicated in detergent (*Decon 90, Decon Laboratories Ltd, East Sussex, U.K.*) for 10 minutes. They were then rinsed thoroughly and ultra sonicated in RO water for 10 minutes, and then ultra sonicated in HCL (1M) this removes any remaining organic matter. They were then rinsed again with RO water and ultra-sonicated in fresh isopropyl alcohol (IPA, 100%) for 10 minutes. for the last time they were then rinsed again in water and then blown dried with argon. Cleaned slides were used immediately.

2.5.3.c Deposition of Metal on Surfaces

For metal deposition the Edwards 306A vacuum evaporator was used. The cleaned glass slides were loaded into the evaporator supported on a stand above the evaporation boats where the gold was placed. (Gold 99.9% Pure, Aldrich [code: 26,579-9]). Once loaded the evaporator was sealed, air was evacuated to create a vacuum with a pressure of $1-2^{-6}$ atm. The first metal deposition layer was Chromium (*chrome plated tungsten rods [code NR1] Megatech, Cannock, Staffordshire, UK*). The chromium was deposited to a thickness of 0.4-0.6nm at a deposition rate of 0.05nm/s. The chromium layer is important as it creates an adhesion layer for the gold layer. The gold layer is then deposited onto the chrome to a thickness of 50nm at a deposition rate of 0.1-0.15 nm/s. Once deposited the slides were left to cool under vacuum for 30 minutes and then removed and stored in a closed polystyrene petri-dish until used.

2.5.3.d Physical adsorption of protein conjugate to create a bio-active sensing surface

The gold sensing surface was converted into bio-sensing surface by physically adsorbing a protein conjugate on the surface. Prior to the physical adsorption of the protein conjugate, the flow cell with the O-ring gasket was assembled onto the sensor. The sensor was first air initiated and then water calibrated. The sensing surface was then washed with NaOH (12N) \ Triton 100X (0.1%) and then followed with water for five minutes. The washing process was monitored to ascertain when the baseline had stabilised indicating a clean surface. The sensor surface was dried with nitrogen while still assembled in the flow cell the sensor was then re-air initiated and water calibrated to establish a background reading were all following measurement would be referred to.

The physical adsorption of ovalbumin conjugated to Estrone-3-Gulcuronide (i.e. OVA-E₁3G) was achieved by flowing past a solution of the protein across the sensing surface. This allows the protein to form a monolayer on the surface with the conjugated analyte adjuncts (i.e. Estrone-3-Gulcuronide) to stick out of the surface to be available for binding to its affinity anti-body partner. A comparison of flow rates and conjugate layer concentration was performed to analyse the optimum method for surface coverage (i.e. maximum. protein adsorption on the sensing surface with very little de-adsorption). The OVA-E₁3G was dissolved in phosphate buffer (PBS) pH 7.4; concentrations of 10, 25, 50, 75, 100, 125,150, and 200µg/ml were used. The sensorgram traces for each solution were compared for protein mass deposition after 10 minutes using the following procedure.

- Sensor assembled and surface cleaned and calibrated
- PBS allowed to flush over the surface for ten minutes before time scan is set (flow rate 10µl/min).
- At 5 minutes after time scan started the conjugate is injected into the sample loop (100µl) and the buffer is switched over to the protein sample.
- The adsorbed protein layer was passed over the sensing surface for 10 minutes and then rinsed with PBS for a further ten minutes to remove any loosely bound protein from the surface.
- The new baseline refractive index measurement was then recorded.

This procedure was performed in triplicate for each of the conjugate protein solution. Analysis of these results determined that 50µg/ml of OVA-E₁3G was the optimal coating solution for the physical adsorption layer. The procedure detailed above optimised the concentration of OVA-E₁3G physically adsorbed within the time parameter of 10 minutes deposition time. The SPR traces were analysed for maximum refractive index change. A desired surface coverage of the protein conjugate at 50µg/ml was achieved between three and five minutes

2.5.3.e Chemisorption of self assembled molecules (SAM) on gold sensing layers

Sulphur containing compounds can spontaneously chemo-adsorb on gold surfaces. 2- mercaptoethanol [sigma M6250] and 11-mercaptoundecanoic acid (MUA) [code: 0450561] were individually used to surface modified the Spreeta™ gold sensing surface. After washing and preparing the gold sensing surface as mentioned above (*section 2.7.2.1*) the sensor was completely taped up using scotch tape leaving only: the sensing surface exposed. The gold sensing surface was then completely covered by pipetting 1ml of 0.1M solution in ethanol (99.9%) of either 3- mercaptoethanol or Mercaptoundecanoic acid. The immersed surface was left over night in a closed Fluoware container to avoid evaporation. The surfaces were then rinsed with pure ethanol and then dried with argon. The sensors were then kept within a dried desiccator until used.

2.5.3.f Covalent attachment of carboxylic dextran to the sensor surface

The mercaptoethanol modified gold sensing surface was further modified by chemo-adsorption of 3-aminopropyltrimethoxysilane (ATPS) to introduce amine-terminated groups to the gold sensing surface. A glass desiccator was taken and 100g of self-indicating silica desiccant (Merck code: 30062) were placed in the bottom and a small beaker filled with 5ml of APTS (sigma code: A3648) was placed in to the middle of the silica desiccant. The prepared clean sensors were supported on a steel mesh above the APTS beaker. This allowed the entire sensing surface to be exposed to the APTS vapour. The glass desiccator was placed under a fume hood and left to evaporate at room temperature. This was left over night and then flushed with nitrogen before the sensor was removed. Carboxyl methyl dextran 0.1% wt/vol (CMD, Avg. Mol Wgt: 600, 000, carboxyl: glucose ratio 1:3) [Obtained from Fluka code: 27560] was dissolved in water and pre-activated with the addition of 1-ethyl-3- (3-dimethylaminopropyl) carbodiimide (EDAC) at 10mg/ml and 5 mg/ml of N-hydroxysuccinimide (NHS). Taking 20µl, the activated solution was

immediately pipetted onto the gold sensing surface and left for an hour in a fluoware container to avoid evaporation from the sample spot. After incubation the sensing surface was rinsed with water (*Weston et al., 1999a*).

2.5.4 Regeneration Protocol

After physical absorption of the protein conjugate and/or subsequent antibody binding events the sensing surface was regenerated by flushing the surface for ten minutes with 1% Persil biological detergent in water (100 μ l/min). The regeneration buffer was then switched to water to rinse the surface of any adsorbed detergent for a further 10 minutes. The refractive index change was monitored at different stages to assess the cleaning process. After cleaning, the surface was available for a new protein adsorption layer.

The investigation and optimisation of different regeneration buffers was performed on the Biacore® 3000. The physical adsorption and regeneration protocol was the same as above. Three different regeneration buffers were investigated: Persil (1%); NaOH (12N) / Triton X-100 (1%) pH 13; Glycine (10mM)/ SDS (1%) and HCL at pH 2.7 were used. PBS and water were used as control buffers. Using J1 plain gold chips the regeneration protocol was followed as detailed, this was carried out first with a plain gold surface and then with 50 μ g/ml ovalbumin protein adsorbed on the surface. The regeneration cycle was performed ten times. The programmed protocol used for the Biacore® experiments are detailed in the table below (*see table 2.1*).

Table 2.1: The Biacore®3000 Programme for protein adsorption and Surface regeneration

Regeneration Programmed Protocol (Biacore ®3000)	
Running Buffer	PBS
Read point:	170 sec.
Water baseline:	Inject - 30 µl @ 180sec
Read point:	350 sec.
Running buffer:	Pass for 300 sec.
Read point:	530 sec.
Protein:	Inject -100µl @600sec
Read point	1430 sec.
Regeneration buffer:	Big inject 150µl @ 1500 sec
Read point	2330 sec.
Water wash & baseline:	Big inject 150µl @2700 sec.
Read point	3130 sec.
Running Buffer:	PBS
Read point:	3410 sec.

Flow rate was set at 10µl/min and data analysis was performed using the BIA evaluation software. To determine weather the physically adsorbed bioactive surface was able to bind αE₁3G antibody. The above protocol was repeated and slight modified by the injection of 100µl of αE₁3G at 1450 seconds, with a read point at 2050 seconds. This would then delay the following procedures by 600 seconds each.

2.5.5 Assay procedure for the portable analyser

The gold surface of the sensor was cleaned prior to performing an assay with the regeneration buffer for 10 min and then washed for a further 10 min. This was important especially when using a new sensor after extended dry storage. Once washed the sensor was then dried with nitrogen gas stream prior to performing sensor initialisation. A typical procedure for performing an assay cycle was as follows:

Before commencement of an experiment the sensor was initialised in air and then calibrated in water to establish a background reading where all following measurement would be referred to;

- PBS running buffer was flown past the surface for 5-10 minutes to obtain a baseline (10µg/min)
- Ovabumin-E₁3G conjugate (100µl) at appropriate concentration was injected into the sample loop and then allowed to pass over the sensor surface after a PBS baseline was established.
- 900µl of sample was pre-incubated with 100µl of anti body 2.5µg/ml for 15-20 minutes.
- The proceeding sample solution was then injected into the sample loop and allowed to flow past the sensor surface at a rate of 10-60µl/min for ten minutes.
- PBS again was then allowed to pass over the surface to remove any unbound antibody from the surface for a further 5 minutes to obtain a baseline measurement.
- The surface was then cleaned/ regenerated by flowing past regeneration buffer for 20 minutes followed by water for further 10 minutes.

Once the assay procedure was completed the cycle was repeated for the next sample measurement. Air initialisation and calibration in water was not required for subsequent measurements.

2.5.6 SPR immunoassay detection and data analysis

The amount of anti-body binding to the sensing surface was found by determining the RI change between the protein conjugate baseline (B_o) and the bound antibody baseline (B). Non specific binding (NSB) was accounted for by subtracting from the immunoassay sensorgram the refractive index binding sensorgram of the antibody to a surface that had only ovalbumin physically adsorbed on to it. The formula used for the calibration graph is given below:

$$\frac{B}{B_o} = \frac{[B - NSB]}{[B_o - NSB]}$$

The SPR calibration curve was plotted as B/B_0 vs. $\text{Log}_{10} [E_13G]$ and was fitted using a four parameter logistic concentration response equation:

$$y = \left[\frac{a - b}{1 + \left(\frac{x}{c}\right)^d} \right] + b$$

This was used to fit the concentration response curves to the data sets (*Sigma plot version 2.00, Jandel Corporation*). Where (a) is the maximum refractive index baseline value of antibody binding, (b) is the minimum refractive index value of antibody binding; (c) is the concentration midpoint producing 50% of the refractive index value of the maximum refractive index baseline and (d) is the slope at the inflection point of the sigmoidal curve. The working range of the assay was defined by 10-90% of the B/B_0 signal, the test midpoint is 50% of the B/B_0 and the limit of detection was determined to be three times the standard deviation of the blank sample (*Mallat et al., 2001*).

2.6 Results and Discussion

2.6.1 Construction and characterisation of the field analyser

The main objective of the project was to develop an analyser that could be used in the field, near the sample source and operated manually or remotely. The analyser was primarily to be used for the detection of endocrine disrupting compounds (EDC) in surface and wastewater environmental samples at wastewater treatment plants or near rivers or streams where effluent discharge from water treatment plants occurs. The Spreeta™ was used, as it is a small, miniature and robust sensor suitable for integrating into custom made equipment. Figure 2.11 (a+b) shows the external and internal view of the constructed field analyser.

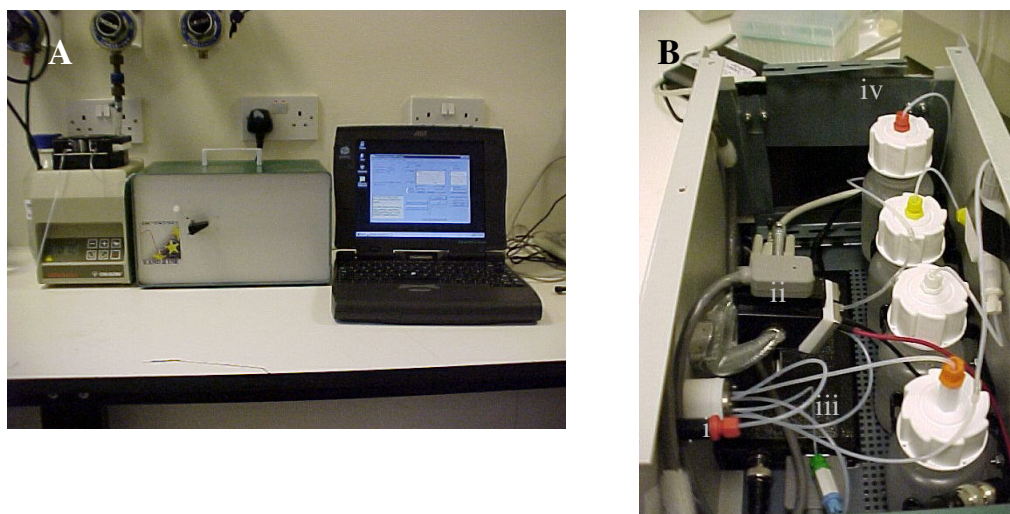


Figure 2.11(a+b): External and internal view of the potable SPR field analyser: a) External view; showing external peristaltic pump and control notebook computer. b) Internal view showing sensor flow-cell connected to sample valve and reagent bottles. (i) Manual sample port with sample loop (100 μ l), (ii) Spreeta™ sensor and connections, (iii) Micro-control box, (iv) Reagent bottles (water, PBS, regeneration buffer and waste).

The manual injection loop had a total internal volume of 200 μ l, with a sample loop volume of 100 μ l. The total volume within the length of tubing from sample loop to sensor was 100 μ l. The flow cell volume is approx. 10 μ l and consisted of an O-ring fitted into the recessed cavity of the sensor. The

constructed self-contained system was easily portable and provided a controlled experimental environment for performing assays. An external thermometer and humidity sensor was also incorporated. The constructed analysers made fluid handling easier and analysed results more reproducible. The weight of the analyser with full reagent bottles was approximately 0.7Kg.

2.6.2 Characterisation and validation of refractive index measurements

The system was initially tested to determine and validate the device for measuring the refractive index of samples. It was found that the Spreeta™ was a very effective tool for measuring refractive index solution and was able to resolve RI changes of sucrose solution down to 0.1% (wt./vol). *Figure 2.12*; shows the progressing shift towards the right of the SPR dip of the Spreeta™ sensor with increasing sucrose concentrations.

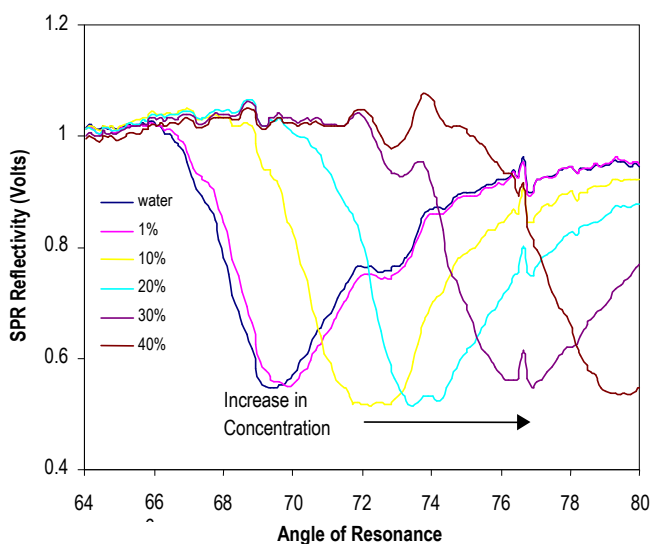


Figure 2.12: Typical SPR minima dip progression with increasing refractive index standards. By increasing the concentration of sucrose solutions the SPR dip angle shows movement towards the right. The reflectivity of the SPR dip remains relatively the same.

The relationship between the refractive index and the concentration of sucrose solution applied to the sensor as seen in *Figure 2.13* showed a very good linear correlation. The measured refractive index of each sucrose solution by the sensor was also comparable to the standard refractive index standard chart

given by Cargille (http://www.cargille.com/hand_brix.html). The equation of the fitted line on the graph is $y=0.0016x + 1.3155$ with R2 value of 0.98.

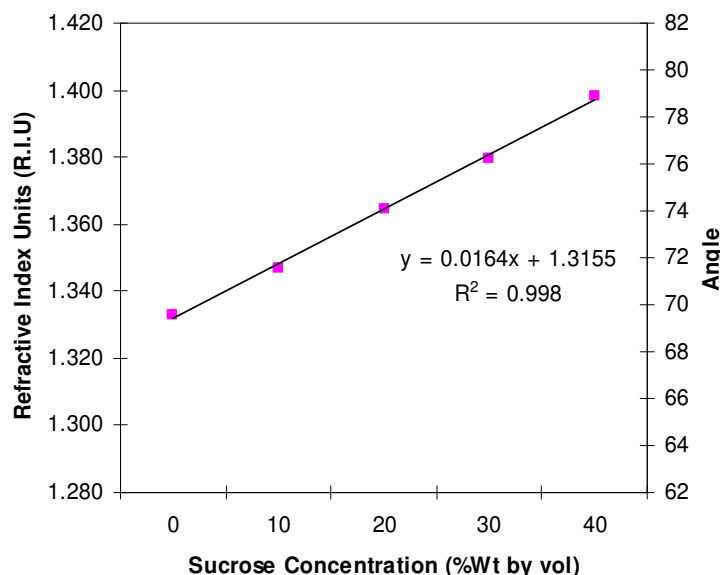


Figure 2.13: Validation of the Spreeta™ sensor with refractive index standards. To validate the sensor as a refractometer known concentrations of sucrose solution were prepared and measured. A very close and accurate correlation was found between the sucrose concentration and the refractive index measured. The linear fitted equation line being $y=0.00164x + 1.3319$ and R2 value of 0.99 .for refractive index units. The SPR angle corresponding to its refractive index unit is shown on the right axis.

The Spreeta™ analyser showed very good linear correlation when plotted against measurements taken with the Biacore® 3000 SPR system using the same sucrose refractive index standards on a plain J1 gold chip (*please refer to figure 2.14*). The concentration of sucrose solutions (i.e. 0.1-15% wt/vol) represents the typical bulk refractive index change of protein concentrations used in immunoassays. From this study it was found that the refractive index resolution of the Spreeta™ sensor is very similar to that of the Biacore® 3000.

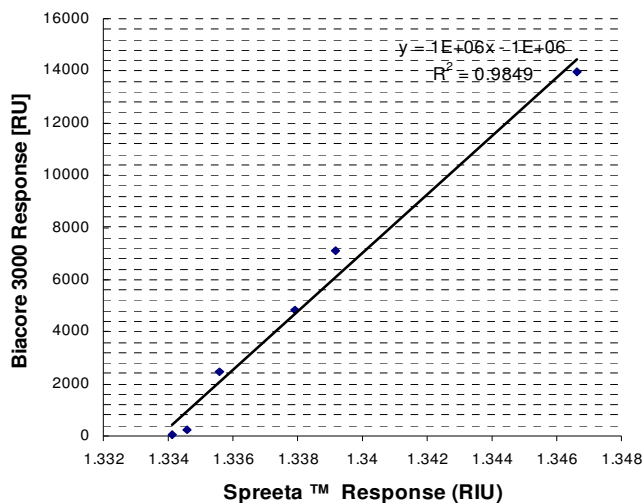


Figure 2.14: Signal comparison between Biacore® 3000 and Spreeta™ SPR devices. The two systems are compared using several different sucrose solutions of 0.1, 0.25, 0.5, 1, 2.5 and 15 (%wt/vol.) in PBS. The linear fitted equation line being $y=1 \times 10^6 x - 1 \times 10^6$ with a R^2 value of 0.9859. Each data point represents the mean of samples taken in triplicate (error bars are too small to be visualised at this scale).

2.6.2.a Short term noise, baseline drift and sensitivity to bulk index changes

The figure given below (figure 2.15) shows an example of the type of time trace sensorgram that is displayed using an 8-bit resolution micro control unit. The short-term noise envelope was 1.91×10^{-6} with a noise level of 3.4×10^{-6} (1 STD), using the temperature compensated dated. This result was comparable to the noise level resolution given in the literature for the analogue digital box.

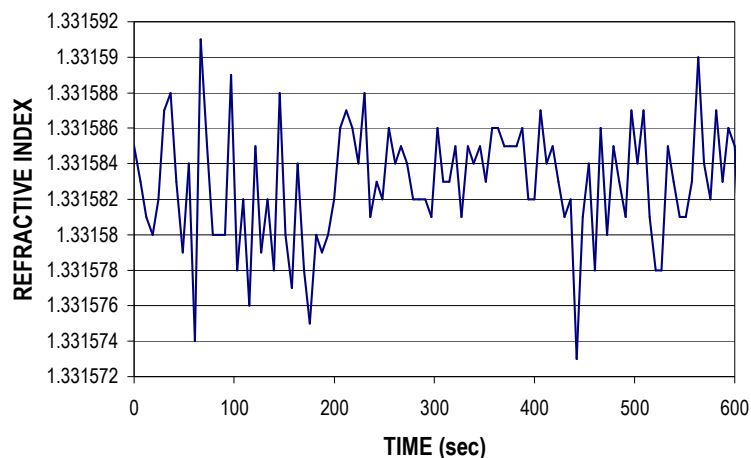


Figure 2.15 Time trace sensorgram of water showing the short-term noise. The noise envelope is 1.91×10^{-5} . The standard of deviation is 3.35044×10^{-6} (3.4×10^{-6}) with the average temperature of 20.00891 stdev of 0.008346. The volume flow through the flow-cell was 100 μ l/min, using the first moment analysis method.

It was observed that the Spreeta™ sensor showed long-term temperature drift, which agreed with, published data of 1.72×10^{-4} over a course of 12 hours. This long-term drift is due to the refractive index change with temperature. Fluctuation changes in temperature can cause significant changes in the refractive index measurements of aqueous solutions ($dRI/dT \approx 1^{-4}/^{\circ}\text{C}$). The temperature not only affect the refractive index of solution but the intensity and wavelength distribution of the sensor's LED, dark current (emission of a small signal even in the absence of light, mostly due to thermal activity in the photocathode and the dynodes) and quantum efficiency of the photo diode array. Short-term drift was observed to mask long-term drift this was in agreement to findings in the literature (*Kukanskis et al., 1999*). The temperature compensated data is calculated as $-.0001$ (R.I.U/Deg $^{\circ}\text{C}$). The table given below (*please refer to table 2.2*) shows the short-term refractive index change due to temperature. All buffers and sample used were brought to room temperature before use to minimise this temperature effect as well as using the temperature compensation data.

Table 2.2: Summarised collected refractive index data and temperature compensated data.

Data given for time trace of PBS flow pass the sensor surface for 10 min at 10 μ l/min (n=7)

SPR Sensorgram of PBS	Refractive Index	Refractive Index (Temperature Compensated)
Refractive index (mean)	1.33466548	1.3346672
STDEV	1.87352 ⁻⁶	1.8255 ⁻⁶

By using the temperature compensation data and equilibrating the fluids at room temperature before flowing pass the surface of the sensor, the sensitivity was found to be 3.1×10^{-7} Refractive index units. The standard deviation is in the order of 0.00001 differences and as long as the samples and sensor is kept at a relative stable temperature, refractive index changes due to temperature could be minimised or avoided.

2.6.2.b Quest in finding the appropriate analysis method

The Spreeta™ evaluation software has seven analysis methods in which the SPR minima dip is found or monitored. Five of these methods were analysed and compared to determine the most appropriate method to be used for further work. Table 2.3 gives a brief description of the five methods used and the characteristic traits in terms of use and signal to noise.

Table 2.3: Software analysis methods and comments

METHOD	Detects SPR Dip Minimum	Comments
(A) First Moments	NO	Only requires a single variable; Fast analysis, Lowest noise
(B) 4th Order Polynomial at specific Value	NO	Can extend the sensor's index range; relatively low noise
(C) Polynomial Fit of resonance minimum	YES	Sensitive to points included in fit; Relatively low noise
(D) Zero dot Product	YES	Relatively low noise
(E) Dot Product of Data with derivative	NO	Measure index changes only; relatively low noise

The evaluation for determining the appropriate method was conducted by measuring PBS buffer with a flow rate of 10 μ l/min, past the sensor. The analysis was initially taken by using method (A): First moment method, the data obtained was saved and re-used to analyse the other four chosen methods. The summarised data given in *table 2.4* showed that the first method is indeed the method that displays one of the lowest amount of noise while performing a timed sensorgram. Method (E) dot product of data showed the least signal to noise standard deviation of the data as well as a smaller noise envelope. Information given in the literature for this method states that the method gives a low noise read out which is equal to but not better than the first moment analysis. The Dot matrix method showed the least amount of noise in terms of its noise envelope and standard deviation. However, the method only measures refractive index changes and requires that a specific RI is given initially before a time scan can be performed. Therefore, would not be suitable for a real time immunoassay monitoring.

Table 2.4: Five software analysis methods refractive index data
for each method its corresponding measured refractive index, noise envelope and standard deviation is given

	ANALYSIS METHODS				
	A	B	C	D	E
Average (RI)	1.336597	1.340793	1.336085	1.336066	1.335589
Noise Envelope	8.42×10^{-6}	1.04×10^{-5}	1.14×10^{-5}	1.08×10^{-5}	1.19×10^{-7}
STDEV	2.11×10^{-6}	2.40×10^{-6}	2.12×10^{-6}	2.52×10^{-6}	6.05×10^{-8}

The first moment of reflectance analysis method was chosen to be used for subsequent assay development. Although it performed second to the dot matrix method it proved a fast analysis procedure and by manipulating the amount of data points to be averaged and choosing a 13 point smoothing tool the noise envelope was significantly reduced.

2.6.2.c Flow rates and refractive index stability

SPR is sensitive to fluid flow velocities as it affects the bulk refractive index of the fluid that is in contact with the sensing surface. This is due to the local temperature fluctuations of the passing fluid occurring at the sensing surface. It was therefore, felt necessary to determine an appropriate flow rate to be used in subsequent analysis. To do this a time trace sensorgram of PBS was passed over a clean sensor surface and for comparison a sensor that had ovalbumin (OVA) physical adsorbed on the surface (prepared by passing $50 \mu\text{g/ml}$ of BSA for 10 min for 15 minutes) at five different flow rates. The short-term noise envelope and standard deviation results obtained were compared (*please refer to figure 2.16a+b*).

The results indicate that the two different surfaces displayed completely different profiles. The short-term noise is greatly reduced once there was a physically adsorbed layer on the sensing surface. Flow rates between $10 \mu\text{l} - 60 \mu\text{l/min}$ was used for subsequent analysis as the short-term noise level was sufficiently low and desirable for use when considering immunoassay sample consumption.

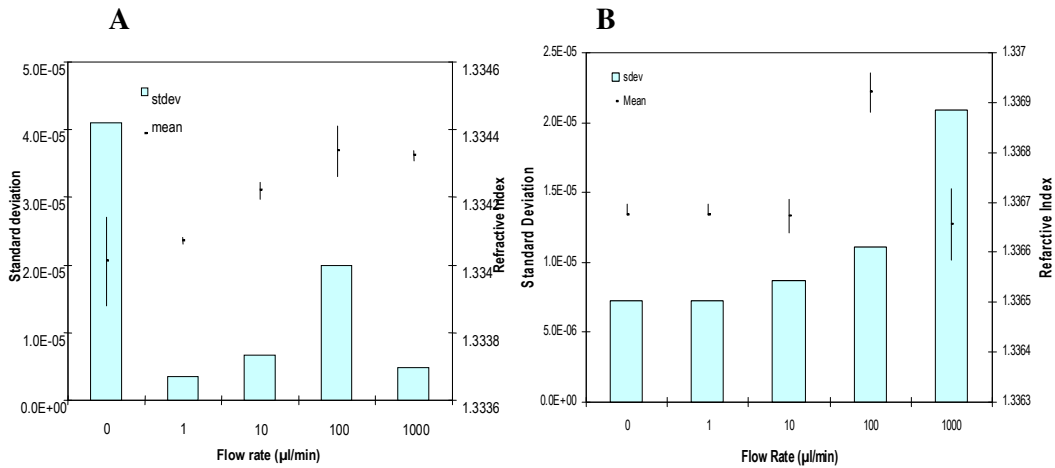


Figure 2.16: (a+b): Flow rate comparison chart. A) Plain gold surface. B) OVA physical adsorbed on the surface. The chart is showing the short-term noise in relation of the flow rate of PBS buffer on a plane gold sensing surface. The line through the mean value links the highest value and the lowest value determined in the period of 15 minutes.

2.6.2.d Summary

The sensor is very sensitive to small changes in bulk refractive index and was found to have a system baseline noise of 1.8×10^{-6} , under the chosen parameters. The refractive index resolution of sucrose at different concentration showed a good linear correlation with data obtained with the Biacore® 3000 instrument. The first moment analysis method was chosen as it displays low noise and fast analysis compared to the other methods. The flow rate between 10 and 60 µl/min was chosen for subsequent analysis as it showed a low signal to noise ratio. Surface modification on the sensor surface reduces the noise level therefore increasing the signal to noise ratio.

2.6.3 Sensing surface preparation and modification

One of the main goals of this research was to find a reproducible and practical method of immobilising a biological specific active layer on to the gold sensing surface and thereafter to find a regeneration protocol that would be appropriate for the performing a semi-continuous immunoassay in the field. Several surface modification procedures were assessed: self assembled mono-layers (MEth, MUA and APTS), thin dextran layers (CMD) and protein adsorption. The main requirement of the surface preparation was that it would be stable, reproducible, low cost and fairly easy to perform in remote situations. The choice of surface immobilisation technique were further limited by the sensors design, as the Spreeta™ sensor used in this study had a reset gold sensing surface which meant that the use of cover slips was virtually impossible hence the gold sensing surface was irreplaceable. The two approaches taken were: (1) by physically adsorbing an active protein layer on the surface to provide a specific bio-layer and (2) by covalently attaching a dextran hydrogel layer on the surface which would then provide a modified surface similar to the sensing matrix used on Biacore chips.

2.6.3.a Cleaning of the sensor surface

It was observed that the pre evaporated gold sensing surface of the Spreeta™ sensor after long periods of dry storage required cleaning. Several cleaning protocols were investigated and the IPA/ HCl (1M) wash was determined to be suitable. Monitoring the sensorgram baseline with water for a period of 20 minutes and monitoring the baseline drift determined this. Before using a new chip the sensor was washed with NaOH / Triton X100 this allowed the sensing surface to be clean and rendered hydrophilic.

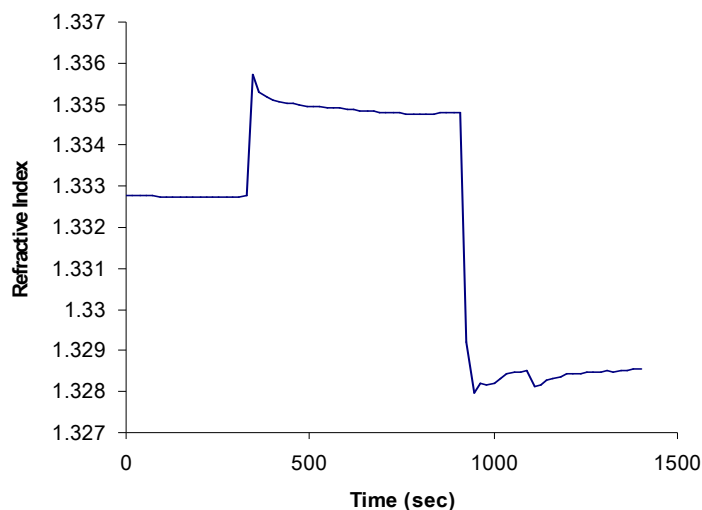


Figure 2.17: Gold surface cleaning of the Sensing surface after dry storage. The sensorgram show the removal of surface adsorbed material from ambient contamination.

The drop in refractive index and the establishment of a new baseline shown in the above figure (*figure 2.17*) demonstrates that the gold surface requires cleaning before use to safeguard against baseline drift issue due to the removal of unwanted material on the sensing surface.

2.6.3.b Determination of appropriate concentration of protein for physical adsorption of the bio-active layer

To determine the appropriate protein concentration for the physically adsorbed protein-EDC analyte conjugate layer, different concentrations of OVA-E₁3G was passed over a clean sensor surface and the adsorbed layer coverage was observed over a period 10 minutes. By physically adsorbing different concentrations of protein over the surface the adsorbing layer maximum surface coverage could be determined. It was determined that 50µg/ml of OVA-E₁3G showed the best monolayer surface coverage. Initial antibody concentration determination for Estrone-3-Gulcuronide was determined by investigating the level of binding of the antibody to the prepared surface using

different antibody concentrations, and concentration of 0.25 μ g/ml of antibody was found to be the optimum concentration of antibody giving a high binding response but low enough for detection of analyte at sub pbb levels.

2.6.3.c Physical adsorption, surface modification and regeneration protocols

As it was necessary to establish a regeneration protocol that was able to effectively strip the gold sensing surface of the adsorbed conjugated protein after performing an immunoassay several buffers were examined. By employing the use of the Biacore® 3000, different regeneration buffers could be screened for their effective surface cleaning. The Biacore®3000 with its advance liquid handling system allowed for direct comparison of the results. The Biacore® 3000 protocol was set up to represent the Spreeta™ analyser parameters as close as possible. J1 Biacore chips were used with PBS buffer pH 7.4 (*see figure 2.18*).

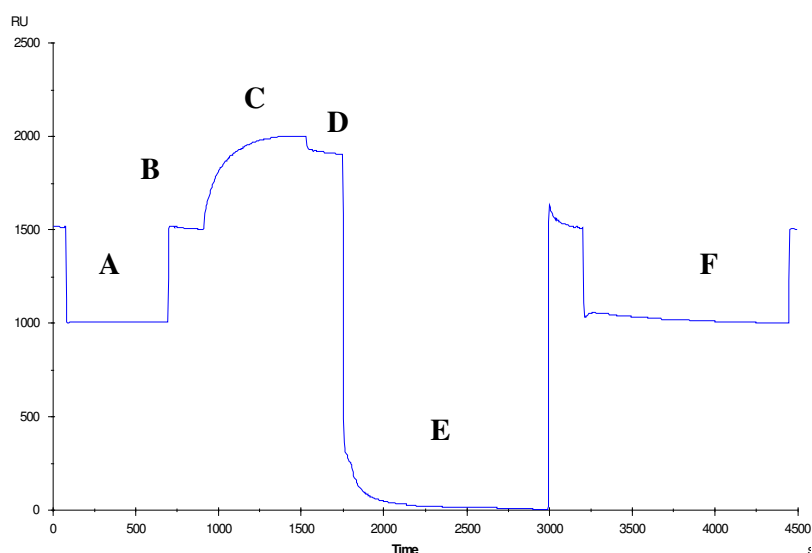


Figure 2.18: Shows a typical regeneration sensorgrams profile of the regeneration protocol carried out on the Biacore® 3000. (A) Water Baseline, (B) PBS baseline-before protein adsorption (C) protein adsorption and baseline, (D) PBS baseline after protein adsorption (E) Regeneration buffer stripping the surface, (F) Water baseline.

The regeneration cycle was performed in such a way as the water baseline was used to monitor the efficiency of the regeneration/ stripping procedure. It was also noted that the final surface rinsing with water removed any adsorbed material especially with Persil. Without the additional water-rinsing step, Persil regeneration buffer was found to increasingly leave a layer of residue on

the sensing surface, this was seen as a rise in the refractive index of water and PBS baseline (*please see figure 2.19a*).

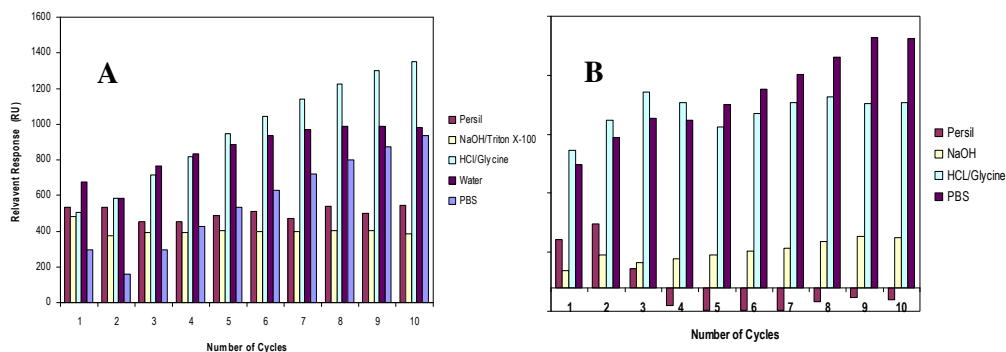


Figure 2.19 a+b: Regeneration buffer relevant response baseline after surface stripping with regeneration buffers on JI gold Biacore Chip. (A) On a bare gold sensing surface. (B) Cycle as above but without protein adsorbing step. Water and PBS were used as control buffer solution. The relevant response represents the PBS baseline after regeneration with different regeneration buffers. Graph (A) shows surface adsorption build up on the surface with PBS, glycine/SDS-HCl and water as indicated by the increase of Relevant RI response. Persil and NaOH/Triton X100 regeneration buffer showed no surface residue build up on the surface after 10 cycles. Graph B shows the protein stripping capability where PBS and glycine/SDS-HCl does not remove any physically adsorbed protein and a build up occurs with each cycle progression.

It was observed that 1% Persil buffer and NaOH/Triton-X100 (0.1%) performed well under these conditions. The NaOH/Triton X100 (0.1%) was a buffer previously used in several publications on Spreeta™ sensor (*Elkind et al., 1999; Strong et al., 1999; Melendez et al., 1996*). The regeneration buffer was used to regenerate the surface but without removing the physical adsorbed protein layer which they considered to be permanently adsorbed. The regeneration of the antibody for NaOH/Triton X100 was very good and the standard of deviation low. However, the binding of the antibody was not as high as that obtained using the Persil buffer and a fresh layer of physically adsorbed protein for the biological sensing layer. The stripping of protein off the surface using Persil regeneration buffer (*see Figure 2.20b*) showed a drop in the baseline refractive index. This maybe caused by the removal of a layer of oxidised gold from the sensor surface, showing a negative relevant baseline value in relation to the original baseline value. However, subsequent antibody and conjugate adsorption layer relevant response values showed no obvious deterioration in the signal level.

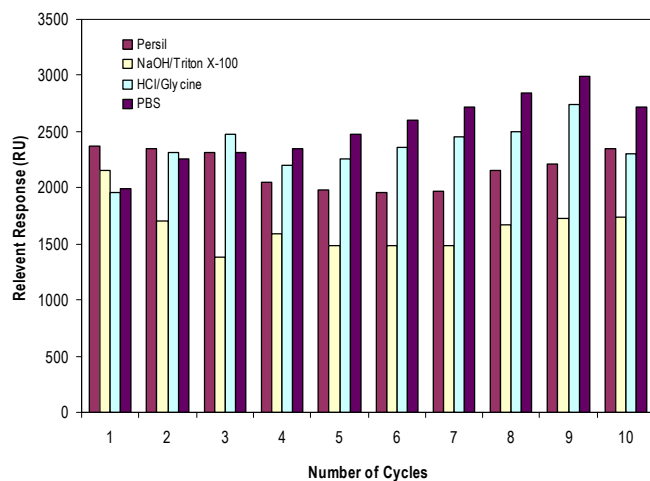


Figure 2.20: Antibody affinity capture baseline after physical adsorption of analyte conjugate protein layer on gold sensing surface after surface regeneration using different buffers. The graph shows the refractive index level of antibody (10 μ g/ml) capture by the newly prepared surface. The affinity capture of antibody for the Persil and NaOH/TritonX100 regenerated surface gives a relatively stable reproducible level where antibody affinity is higher of the Persil regenerated surface than NaOH/TritonX100. For PBS and glycine/SDS-HCl the antibody response increases with number of cycle. This indicates that the surface is not being striped completely after each cycle and a build up of adsorbed protein on the sensing surface is observed.

The standard deviation for antibody binding layer for both Persil and NaOH/Triton X100 were both low, with the antibody baseline for the Persil regenerating buffer being a little higher this suggests that a better ovalbumin-E₁3G surface coverage was achieved (*please refer to figure 2.20*). The glycine /SDS-HCl regeneration buffer performed the least well as no material from the surface was removed, indicated by the increased refractive index response, indicative of a build up of protein on the surface. It was concluded that both NaOH/Triton X100 and Persil regeneration buffer were suitable regeneration buffers for consideration.

The NaOH/ Triton X100 buffer could be used as a regeneration buffer for a single sensor that could be used continuously for one particular analyte with very little deterioration of the antibody binding after fifteen cycles. Melendez

et al. (1999) (Melendez et al., 1996) used this method of regeneration and noted that the sensor surface was reusable after 3 months of the surface being prepared.

Strong et al. (1999) (Strong et al., 1999) also suggested that the surface required a few assay to be performed on the surface as a conditioning step to prepare the surface. This may be due to non-specific binding and weak adsorption of the protein forming bi-layers. Persil on the other hand provide another regeneration strategy that enabled the complete stripping of the surface of the appropriate protein. This would allow an assay protocol of adsorbing differ protein conjugates onto the surface and hence the detection of different analytes sequentially with the same sensor.

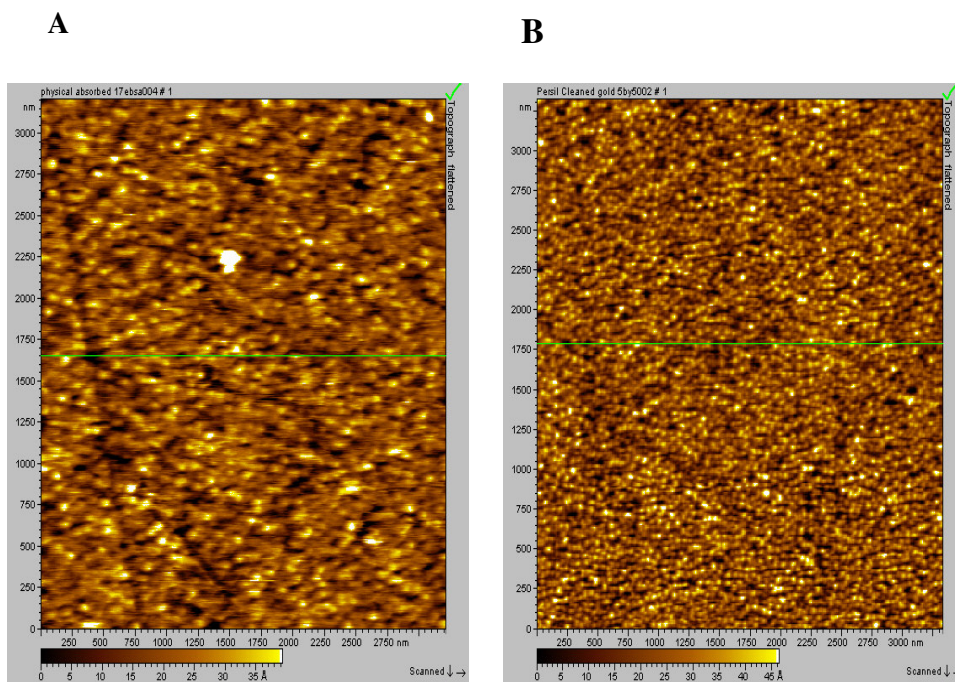


Figure 2.21 (a+b): AFM images of a gold surface with adsorbed Ovalbumin-E₁3G protein layer at 50 µg/ml and the same surface cleaned with Persil (1%). The slightly blurred image in (A) represents the physical adsorbed protein on the surface with slight dragging on scanned material due to AFM tip being in contact with the surface. Picture (B) represents the same surface after cleaning with Persil (1%). The small granular pattern is typical of gold scanned by AFM.

AFM and contact angle study of the surfaces cleaned and regenerated with the different buffer showed that NaOH/Triton 100X left the surface of the gold layer hydrophilic while Persil left the surface more hydrophobic as indicated by the contact angle measurements. The scanned surface of a gold surface with

protein adsorbed on the surface and then washed with Persil did confirm that the surface had been completely cleaned of adsorbed protein (*please refer to figure 2.21*). The scanned picture of the Persil cleaned gold slide to clean unused gold surface slide were comparable. The protein adsorption-regenerating assay was performed on the Spreeta™ sensor using Persil to investigate the use of the regeneration procedure. The results obtained are summarised in *Table 2.4*.

Table 2.4: Demonstration of repeated regeneration of a gold SPR sensor surface after physical adsorption of protein using a commercial laundry detergent

(±ISD, n=15)

Regeneration With Persil	SPR Resonance ANGLE (°)
Water Baseline	69.158 ± 0.0164
PBS Baseline	69.340 ± 0.063
Conjugate Baseline (in PBS)	69.356 ± 0.0045

The small average increase due to adsorption of the OVA-E₁3G conjugate is due to the de-naturalisation of the protein upon adsorption. The variation of the SPR angle for the various steps in the 15 repeats demonstrates a significant level of variability in the absolute SPR angle. However, this also coincides with the variability of the system baseline noise. Therefore, relevant changes within cycles are not as significant and proportionally reproducible.

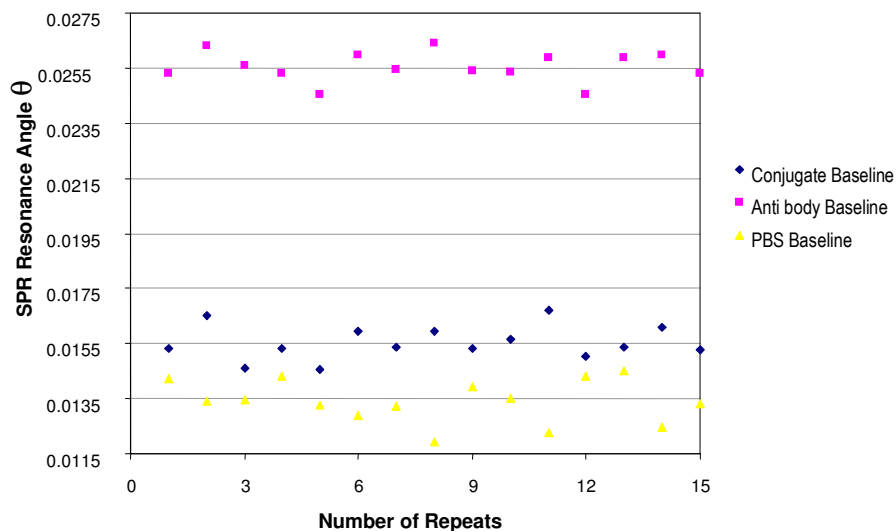


Figure 2.22: Relevant baseline of conjugate protein, antibody and PBS. Data points represents the baseline after 15 sequential repeats of adsorbing ovalbumin-E₁3G on the sensing surface, binding the antibody and then regeneration of the test format with Persil (1%) before a new cycle is performed.

The above figure (Figure 2.22) shows the typical assay procedure undertaken with the Spreeta™ sensor analyser. The baseline of PBS buffer, Ovalbumin-E₁3G protein conjugate and antibody binding was monitored to determine the repeatability of an antibody binding assay and efficiency of the regeneration process of the surface using Persil. This was performed by adsorbing the appropriate protein on the surface and removing all analysis samples from the surface, starting a fresh each time. The results show after 15 sequential assays the repeatability of was very close.

2.6.3.d Summary of surface modification and regeneration

The immobilised active biological specific layer for the sensing surface can be achieved by an *in-situ* physical adsorption method. Sequential adsorption and regeneration of the sensing surface using Persil (1%) was a suitable regeneration buffer for the assay format used.

2.6.4 Initial immunoassay for Estrone-3-Gulcuronide

2.6.4.a Determination of specific and unspecific binding

To determine the specific and non-specific binding of antibodies to the physically adsorbed OVA-E₁3G conjugate a sample of anti-E₁3G and anti-human chronic gonadotrophin (HCG) antibody was applied to the sensing surface at a concentration of 0.25 μ g/ml. Preparing the surface with pure ovalbumin and conducting the same procedure determined the level of non-specific binding. The determined level of non-specific binding of the antibody was subtracted from the specific binding sensorgram. *Figure 2.23* shows the normalised refractive index sensorgram of blank sample of anti-E₁3G and anti-HCG that was each incubated at the sensor surface for 10 minute. The relative refractive index measurement ratio has been normalised to the average of the baseline ratio (i.e. representing the baseline for PBS alone) over 150 sec. before sample injection at the beginning of each test cycle.

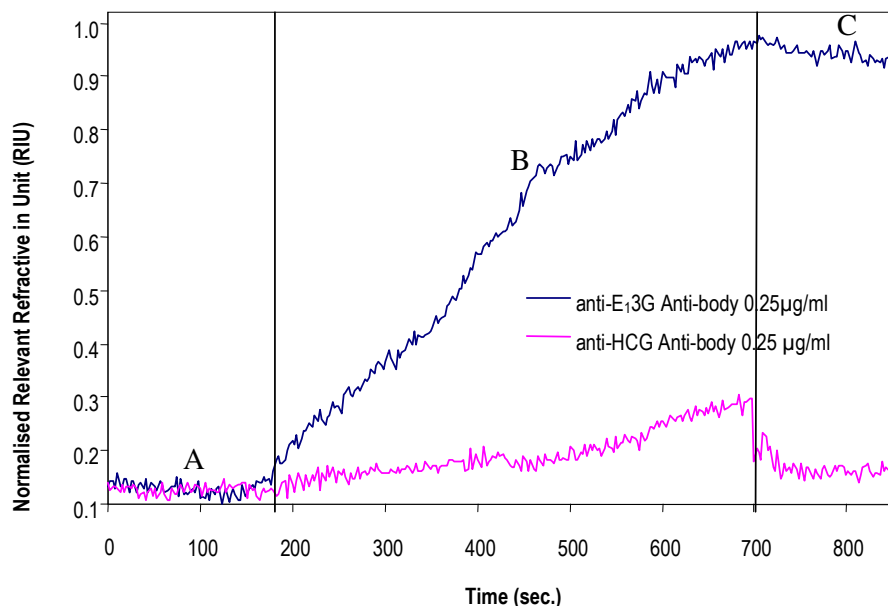


Figure 2.23: Response of specific antibody and non-specific antibody to the sensing surface. A) Represents the baseline for PBS. B) Incubation of antibody to the sensing surface and C) is the new PBS baseline.

The result obtained by measuring specific anti-E₁3G antibody to the adsorbed layer and non-specific anti- HCG antibody shows the specific response for anti E₁3G antibody (0.25µg/ml) to have a refractive index baseline 40 times higher than the PBS baseline. The response of the anti-HCG antibody to the surface shows little binding. However, after PBS is passed over the surface the baseline immediately drops and the new PBS baseline measured is less than 1% higher than the original PBS baseline (*please refer to figure 2.23*) Incubation of <1mg/ml of ovalbumin in PBS at the sensor surface also showed 1% increase in the measurement. This change is equal to the minimum change resolvable by the sensor and show that the sensor is highly specific to monoclonal E₁3G antibody. At high concentration of ovalbumin (1mg/ml) adsorption on the sensing surface leads to a very minor amount of non-specific binding of protein at the sensor surface. Therefore, the Ovalbumin-E₁3G conjugated protein surface is well coated.

2.6.4.b Immunoassay calibration curve

The devised assay protocol was designed and optimised for the detection of Estrone-3-Gulcuronide (E₁3G). *Figure 2.24* shows a representation of the assay time line. *Figure 2.25* shows a typical analysis sensor gram.

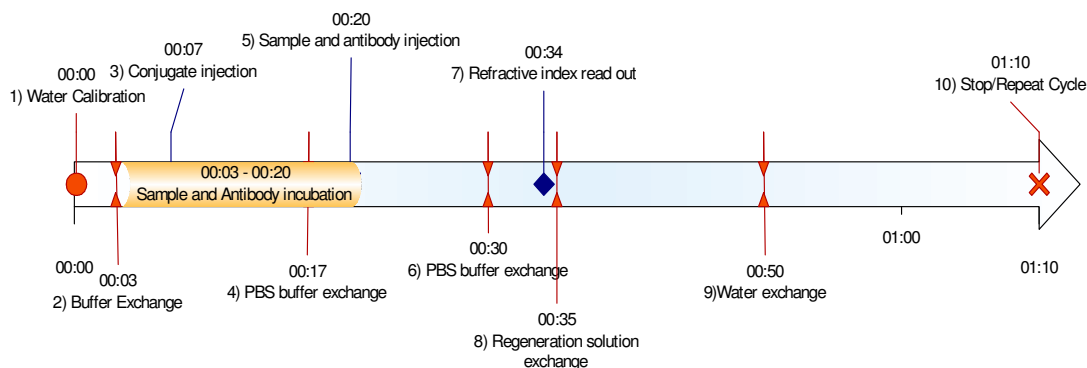


Figure 2.24: Assay cycle timeline performed on the SPR analyser: The assay cycle for in-situ preparation of the biologically -active surface and surface stripping for regenerating the surface is initially performed by: (1) calibrating the sensor in water to obtain a baseline. (2) The water is exchanged to PBS and this is passed over the sensor surface for four minutes to establish a PBS baseline. (3) The protein conjugate is injected into the sample stream and allowed to flow over the sensor surface. PBS buffer is passed over the physically adsorbed protein layer for three minutes to wash any unbound protein from the surface. (4)The antibody and sample is injected into the carrier flow and allowed to pass over the sensor surface. Buffer exchange and timings of assay cycle is given in hours: minutes.

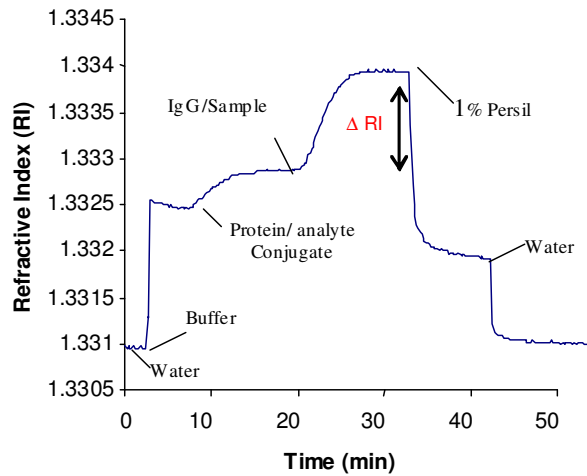


Figure 2.25 Typical Spreeta™ analyser assay cycle demonstrating both sensor regeneration with domestic laundry detergent and measurement of anti body binding. Relative refractive index change is determined by the difference of the baseline of PBS over the physically adsorbed ovalbumin-E₁3G conjugate layer and the new PBS baseline after antibody binding.

A set of sequential binding responses of the immunosensor to whole monoclonal anti Estrone-3-Gulcuronide antibody (0.25µg/ml) pre-incubated with known concentrations of E₁3G is shown in *Figure 2.26*. The relative refractive index measurement ratio has been normalised to the average of the baseline ratio (i.e. representing the baseline for PBS alone) over 150 sec. before sample injection at the beginning of each test cycle. The assay was performed as developed in the assay schematic given in *Figure 2.25*. The full set of data for the assays calibration curve took eighteen hours over a period of three days each concentration were made in triplicate (*please refer to figure 2.6*).

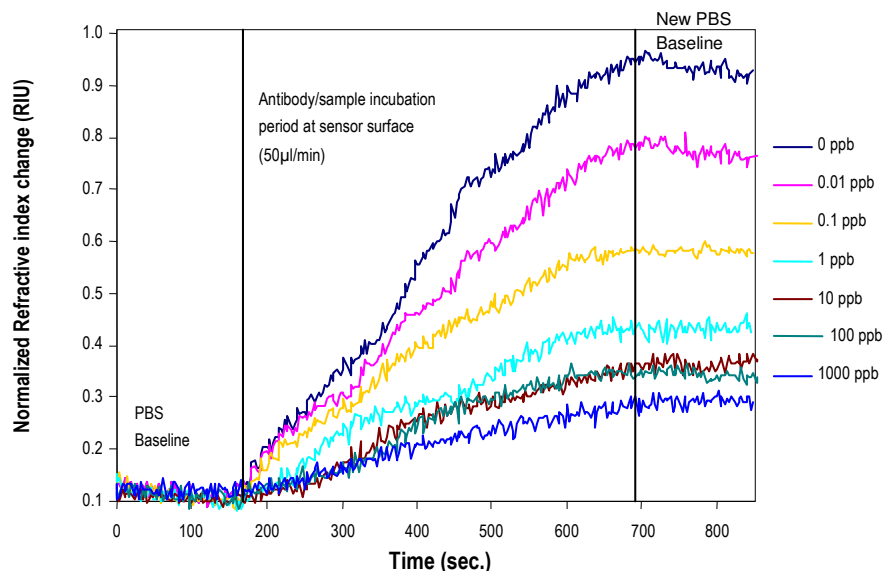


Figure 2.26: A typical real time SPR immunosensor response for several E_13G concentrations over the range of $0-100\mu\text{g/L}$. The amount of antibody attached to the surface after incubation is inversely proportional to the analyte concentration of the sample.

The amount of antibody after incubation that is bound to the sensing surface is inversely proportional to the analyte concentration of the sample. Hence, the gradient of the steepest line represents the binding rate of the antibodies in the blank solution to the sensor surface. The curve in the middle of the plot represents E_13G concentrations below $1\mu\text{g/ml}$ and the smallest gradients represent the concentration of E_13G at 100 and $1000\mu\text{g/ml}$. *Figure 2.27* show the calibration curve of E_13G concentration using monoclonal anti E_13G with a 4 points parameter sigmoidal curve fitted to the averaged data set. Data calibrations sets were performed in triplicate and the error bars on the data point represent one standard deviation of the mean of the averaged data points. The lower limit of detection of the sensor was found from the fitted curve, at the concentration where the measured concentration had fallen to three times the standard deviation below the mean measurement for the blank sample (*Coille et al., 2002; Harris et al., 1999*). The mid point of the calibration curve was determined to be $3\mu\text{g/L}$, whilst the upper limit of the operating range for this assay, represented by the measured value at 90% below that of the blank sample was at $100\mu\text{g/L}$.

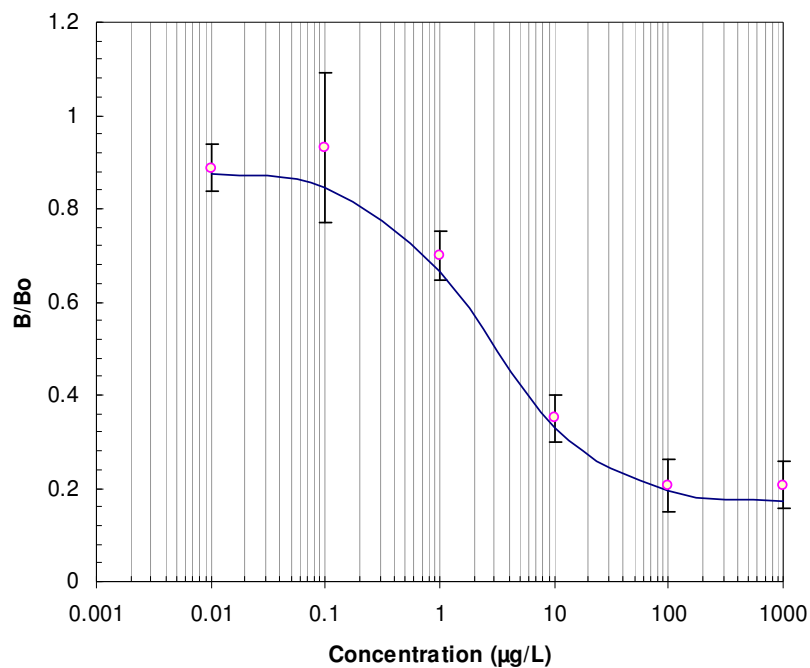


Figure 2.27: Calibration curve for Estrone-3-Gulcuronide with the Spreeta™ analyser. The standard curve is derived from standard samples analysed in triplicate. The error bars indicate the standard deviation (1STD and $n=3$). B is the refractive index baseline of the sample after binding to the surface and B_0 is the PBS baseline before binding.

The working range of the assay was found to be between 1-10µg/L with a limit of detection of 0.3µg/L and the maximum limit at 20µg/L. The calibration curve shows that the devised immunoassay could be used to determine E₁3G concentrations in the sub ppb range.

2.6.5 Discussions and conclusions

2.6.5.a Construction and characterisation of the analyser

The commercial available surface plasmon resonance biosensor Biacore ® 3000 is a fully automated system combining an auto sampler for precision liquid handling and integrated micro fluidics and detection. This allows for the system to be precise, rapid and simple for samples to be analysed. The system is however expensive and desktop bound; the Spreeta TM system on the other hand is a very small and simplistic sensor system. The sensitivity of the sensor is comparable to the Biacore ® 3000 in terms of its signal to noise output this finding was also in accordance to published data (*Leonard et al., 2003*).

Incorporation of the sensor into a self-contained analyser with a fluid handling system provided a platform system that could be used for sample bio-affinity analysis. Initial experimentation and optimisation of the sensor with buffered sample using Estrone-3-Gulcuronide as a model estrogenic compound demonstrated that the sensing platform could be used as an immunosensor with good sensitivity.

2.6.5.b Assay Protocol and Regeneration

The constructed assay protocol design allowed for *in-situ* surface modification of the sensing surface that could be replaced remotely and automatically by a simple combination of fluidics, non-covalent immobilisation and regeneration /cleaning step. To enable the sensor reusability a novel regeneration step using Persil liquid laundry detergent was used. As the gold sensing layer was considered to be irreplaceable a surface stripping method was sort after. Typically, regeneration procedures for affinity sensors commonly involve exposure of the surface to single component detergents, variations of pH, variations of ionic strength etc. (*Andersson et al., 1999*) The novel use of Persil detergent as an alternative was chosen as it contained a defined complex mixture of surfactants, proteases, cellulase, lipases and bleaching agents compared to other traditional recipe approaches.

During the initial investigation for converting the sensor into an immunosensor several issues on optimisation were apparent. Assay time with regeneration of the surface was over 1 hour. The lengthy assay time was mainly due to the

regeneration step of the surface as the actual surface preparation and binding time was only 15 minutes. This could be addressed by improving the fluid handling system and having automated control on varying the flow speed and reagent buffer exchange. A more relevant antibody or receptor against a specific EDC would be desirable, as it could be directly compared to EDCs found in the environment.

2.6.5.c Covalent antigen immobilization

One of the main goals of this research was to find a reproducible and practical method of immobilising a biological specific active layer on the gold sensing surface and a regeneration protocol that would be appropriate for the analyser. Several surface modification procedures were investigated: self assembled mono-layers (i.e. using APTs and Mercaptoundecanoic acid [MUA]), Thin dextran layers (CMD) and protein adsorption. The main requirement of the surface preparation was that it would be stable, reproducible, low cost and fairly easy to perform in remote situations. The choice of surface immobilisation technique were further limited by the design of the sensor as the Spreeta™ sensor used in this study had recessed gold sensing surface which meant that the use of cover slips was virtually impossible.

Physical immobilisation of the antigen conjugated protein proved to be successful. However, the second approach of immobilising the desired estrogenic antigen onto the sensors surface was also investigated. Surface modification by covalently attaching a hydro-gel to the sensing surface was of interest as it would create a sensing surface similar to that of the Biacore® 3000 system where simple water based EDC/NHS chemistry could be used to covalently attach the antigen and also to provide a barrier for non specific binding. The aim was to find a quick and practical way of preparing the surface and so the reverse deposition model (*Weston et al., 1999a*) was proposed. This is where the antigen of interest in this case Estrone-3-Gulcuronide would be covalently attached to the dextran backbone structure and purified before covalently attaching on to the pre animated modified gold surface. Once achieved a chemical and/or biological regeneration protocol was to be sort after and optimised for use in remote conditions.

Covalent attachment of the dextran hydrogel proved to be very difficult as the resolved SPR dip was too shallow to be of any use for refractive index probing. For a good signal, the resolved dip should ideally be below 0.6mV especially if measuring with first movement of momentum analysis technique were the baseline is set preferable at 0.8mV. The resolved dip for the modified dextran surface was above 0.8mV and therefore unable to be used for monitoring.

Surface immobilisation of E₁3G by direct covalent attachment to the APTS and 1% MUA modified surfaces were also investigated and were equally unsuccessful (data not shown). In the literature there are given examples of covalently attached antigen immobilisation to the Spreeta™ sensor that have been successful where they have used gold binding protein (GBP) (*Naimushin et al., 2002; Woodbury et al., 1998*) and also MUA (*Simonian et al., 2002*). The unsuccessfulness of the covalent attachment procedures carried out in this study may be due to the air initialisation data that was used. Initialisation could also be performed in buffer that has a higher refractive index then the region that is being probed i.e. 15% sucrose or alcohol this approach may have resulted in a better response. Due to the unsuccessfulness of the modification procedure this line of investigation was not pursued further.

2.6.5.d Conclusion

The Spreeta™ sensor was successfully integrated into a portable self contained analyser. The devised immunoassay and regeneration protocol that elicit the use of a propriety laundry detergent Persil biological (1%) was used to measure Estrone-3-Gulcuronide in buffered samples. The modification of the sensing surface was obtained by *in-situ* physical adsorption of the conjugated E₁3G to ovalbumin. It was observed that repeated absorption and stripping of the protein from the surface did not have a significant effect on the refractive index change measured for either the protein conjugate level or the antibody binding.

3 Refinement, development and optimisation of an EDC Immuno-analyser

Chapter 3

Refinement, development and optimisation of an EDC Immuno-analyser

3.1 Introduction

In the previous chapter the Spreeta™ SPR sensor was investigated and characterised for immunoassaying application. It also described the initial construction and immunoassay design for the SPR analyser to be used in the field, at source or as a remote sensor. It was determined that the sensor was easily integrated into a portable system and a relatively simple surface modification and regeneration protocol could be performed for the detection of Estrone-3-Gulcuronide analyte in buffered samples. Several issues on optimisation and use of a more relevant model antibody for detection in real environmental samples were highlighted.

3.1.1 Testing pollutants in environmental samples using ELISAs and immunoassay applications

The European community and international agencies has set the limits on the concentrations of environmental pollutants such as pesticides and heavy metals that may exist in ground water, surface waters and waste waters. There is currently no limit of concentration for endocrine disrupters as a group, especially for natural or synthetic estrogenic hormones. However, as many of the xeno-estrogen mimics also have other toxic properties and many pesticides have also been shown to have EDC effects the current legislation limits for pesticides can be used. The limits of concentration for pesticides are 0.1µg/ml for a single pesticide and 0.5µg/L for the combined total of pesticides. However, we know that EDCs, especially estrogenic hormones are present in the field at *ppt* levels. Immunoassays offer a route for rapid *in-situ* analysis for water samples for EDC detection at low cost. Immunoassay based methods are popular because of their low price and rapid *in-situ* analysis coupled with an immuno- affinity reaction, specific analytes can be detected with high specificity and low detection limits.

ELISA test kits for the detection of steroidal hormones are well establish and were initially designed for clinical use and only recently has been evaluated for use in environmental samples. Immunoassay performed on biosensor are often direct transfer of ELISA based assays with further optimisation for that particular transducer and analyte quantification. ELISA can provide useful optimisation information when devising an immunoassay for a biosensor as many tests can be preformed on one plate. The use of an ELISA for detection of environmental samples have also proved to be highly comparable with GC and HPLC detection (*Huang et al., 2001*).

3.1.2 Detection of EDC in waste water and surface waters

Publications of EDC detection in waste water and surface water are limited as much work on this specific area only started in the last six years and the first phase of investigation for European Union Research initiatives was completed in 2002¹. Four recent papers on EDC detection in wastewater have detailed the use of commercial ELISA kits, GC-MS and a fluorescence based optical affinity sensor. (*D'Ascenzo et al., 2003; Coille et al., 2002; Huang et al., 2001;*

¹ This research was undertaken as a component of the SANDRINE project (ENV/ct98-0801) funded by DGXII Environment and Climate (1994-1998) work programme of the European commission.

Espadaler et al., 1997; Aherne et al., 1985). Each author states that they were able to detect their EDC analyte down to sub ppb levels.

3.2 Aims

The aims and objectives of this chapter were several, firstly further refinement and integration of new hardware to the analyser and design development of an automated fluid handling system for the analyser was to be investigated. Monoclonal antibody against 17β Estradiol was to be used as a more relevant model antigen. An ELISA assay using antibody against 17β Estradiol was to be developed and optimised to aid assay development for the Spreeta™ analyser. For further assay development and verification of results the assay was to be transferred onto the Biacore® 3000 platform. The comparison of different sets of results was to give further information on the design of the immunoassay and protocol automation by using a well defined SPR system which has a sophisticated liquid handling system. Lastly the optimised assays (i.e. the in house ELISA, Biacore® 3000 assay, and Spreeta™ analyser assay) were to be used to assay spiked 17β Estradiol in buffered, synthetic wastewater samples and then in real wastewater samples. The results obtained were then to be compared with results from a commercial ELSIA assay kit.

3.3 Equipment, Materials and Methods

3.3.1 Spreeta™ sensor Updates and incorporation into the field analyser

The results represented in this chapter were all conducted using the new Spreeta™ flow cell and 12-bit digital signal processor (DSP) purchased from Nomadics Inc (Stillwater, Oklahoma, USA), the external pump used with the analyser was replaced by a miniature peristaltic pump from Krajci Engineering (Warsaw, Czech Republic) (please refer to figure 3.1 a and b). All other components of the developed analyser equipment were the same as detailed in (section 2.3.4a).

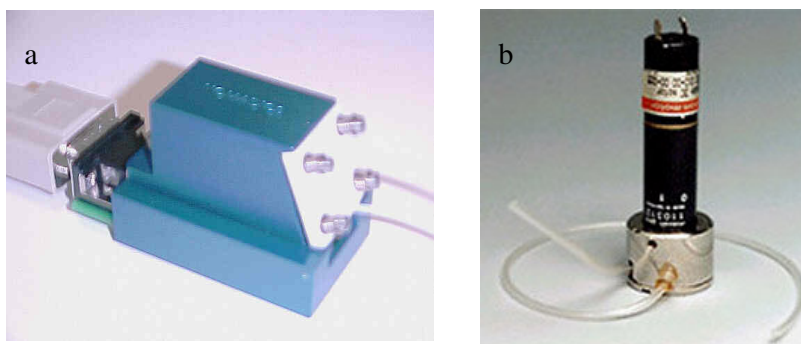


Figure 3.1 a+b: *Photographic illustration of the new integrated flow cell and miniature peristaltic pump. a) The new integrated flow cell, once the sensor is assembled into the flow cell the only other connection required is the control box, b) The miniature peristaltic pump was easily incorporated in to the analyser. The pump was controlled by a separate control circuit and 6 volt battery.*

3.3.2 Biacore System

The Biacore®3000 SPR analyser was used with a flow rate used was 10µl/min using filtered PBS pH 7.4 buffer (0.25µm syringe filter).

3.4 Materials

3.4.1 Biological reagents and buffers

Immuno-reagents anti 17 β -Estradiol-6 (1.0^{10} L/M) was purchased from Fitzgerald, MA USA (clone M94150). Estradiol-6-CMO-BSA (E₂-BSA) conjugate [Sigma code E5630] and Estradiol (E₂) [sigma Code E3346] purchased from (Sigma Poole UK).

3.4.2 Buffers and Reagents

Phosphate buffered saline (PBS) pH 7.4 was used as a running buffer to prime the sensing surface and. RO water was used for buffers and for refractive index calibrations. 1% Persil buffer in water and NaOH /Triton X100 was used as a regeneration buffer.

3.4.3 Preparation of Synthetic Waste Water

The described composition of compounds was given by (Dresden University) and is a modified method based on a standard recipe (Prescript: DIN 38 412 Teil 26). There are two components to the stock solution (A+B), recipe of the solutions are described below

Table 3.1: Solution A: Protein solution

Compound	Supplier	Weight (g)
Casein	Sigma [C7078]	32
Meat extract	BD diagnostics systems [0115-17]	22
Urea	Sigma [U5378]	6
NaCl	Sigma [S3014]	1.4
CaCl \cdot 2H ₂ O	Sigma [C3306]	0.8
MgSO ₄ \cdot 7H ₂ O	Sigma [M1880]	0.4

All of the above compounds were dissolved in 100ml of tap water. The suspended solution was autoclaved for 20min at 121°C. The resultant solution was a clear yellow solution. Aliquots of 10 ml were deep-frozen and were stable in this condition for long extended periods. Solutions once thawed were stored in the refrigerator at 4°C and were stable for 1 month.

Table 3.2: Solution B: Mineral salt solution

Compound	Supplier	Weight (g)
K ₂ HPO ₄	Sigma[P9666]	47

The potassium salt was dissolved in 100ml of RO water and then divided into 10ml portions and deep-frozen. Thawed solutions were able to be stored in the refrigerator at 4°C and were stable in this condition for six months. Solution A and B are concentrated solution.

3.4.3.a Preparation of working Solution of the Synthetic Waste water

The wastewater was prepared by taking 3ml of Solution A and 0.5ml solution B in 1200 ml of tap water (*see table 3.1 and 3.2*). The wastewater was prepared freshly when required as the organic components are easily degraded by biological process.

3.4.3.b Calibration standards and test samples in synthetic and waste water sample

Preparation of test samples in buffered, synthetic wastewater and real sample waster water was the same for all analytical assay applications and was as follows:

Estradiol standards were prepared by dissolving 1 mg of Estradiol in ethanol and diluting down to 1µg/ml stock solution in PBST. The concentrations used to construct the standard curves were between 0.001 and 1000ng/ml

Buffered samples: 900µl of standard calibration in PBS was incubated with 100µl of antibody

Synthetic wastewater samples: 900 µl of synthetic waster was spiked with 100µl of Estradiol calibrate stock solution (10x required concentration). 800µl of the made up sample was taken and buffered with 100µl of PBS (5x normal concentration). 100µl of antibody was then added.

Real samples: Samples were prepared as above mentioned above for synthetic waster water samples.

For all procedure the samples were allowed to pre-incubate between 15 and 20 minutes

3.4.4 Collection of real waste water samples

The real waste water samples were collected from Cranfield University Wastewater treatment works and sample at point 1 and 2 representing in influent and effluent of water going through the treatment works (*please refer to figure 3.2*). All samples were filtered using a 0.45µm syringe filter and then with a 0.25µm syringe filter to sterilise and reduce matrix effect. Samples were immediately deep frozen and thawed over night prior to use. Analyses of the samples were taken within one month of sampling.

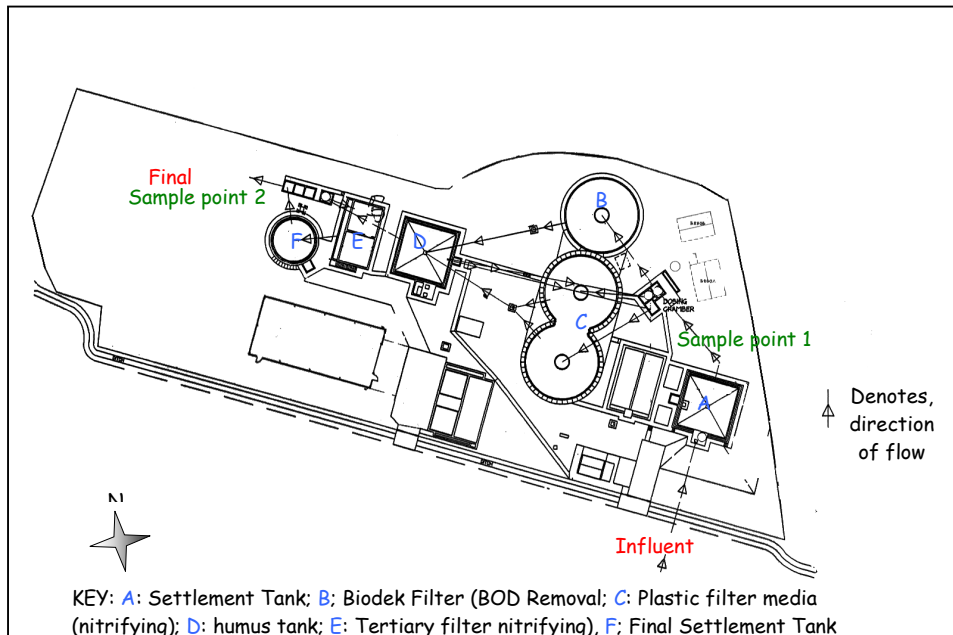


Figure 3.2: Map of Cranfield Sewage treatment Works (Bedfordshire, UK.) Samples were taken at point A and B as indicated on the map. These sites were chosen to represent the influent and discharge of the raw waste water sample and discharge sample that enters into a 8stream represent surface water.

3.5 Immunoassays methods, development, optimisation and protocols

3.5.1 ELISA Development

A checkerboard titration was used to select the working proportions for coating Estradiol-BSA (E₂-BSA) and primary antibody for use in the ELISA. Two fold dilutions of E₂BSA (1000ng/ml to 0.001ng/ml) were prepared in PBS pH 7.4. A row of wells on a 96-well micro-titration plate (Polysorb Nunc) was coated with each Estradiol solution (200µl per well). The plate sealed was then allowed to incubate over night at 4°C. After incubation the coating buffer was removed by flicking out, the plate was then filled (300µl per well) with 1% OVA in PBS and incubated for 1 hour at room temperature to block any non-specific binding sites. After incubation the OVA solution was flicked out and then washed 3 times with PBST (Phosphate buffered saline containing 500µg/L Tween 20). A doubling dilution series of anti-Estradiol was added to the plate (100µl) row wise to the plate. The plate was sealed and incubated for 1 hour at room temperature and then inverted and washed three times with PBST.

Monoclonal anti mouse IgG labelled with horseradish peroxidase (1:15000) was diluted in 0.1% OVA and added to each well (200µl per well). The plate was again covered and incubated for 1 hour after which it was washed three times with PBST. The ABTS ready to use ELSIA substrate was added to each well (100µl), agitated and then left to incubated for 60 minute in the dark. The optical density of the wells was read at 20, 45 and stopped at 60 min with 2M HCL. Absorption reading was taken at 405nm. Combinations of conjugate coating and anti- Estradiol concentrations that yield an optical density (OD) of 0.8-1.2 within 60 minutes were noted. A selection of those combinations was further evaluated through the preparation of ELISA.

3.5.2 Optimised ELISA Procedure

The wells of the 96 well micro titre plate were coated with Estradiol-BSA (50µg/ml), eight wells coated with BSA were left for correcting for non-specific binding (NSB). The coated plate was prepared the day before use and stored at 4°C. Taking the standard stock solution of Estradiol (1000µg/ml) a

dilution series was made up in PBS pH 7.4. Monoclonal anti- 17β Estradiol antibody was diluted to $2.5\mu\text{g/ml}$. Sample test mixtures of analyte standards ($900\mu\text{l}$) and αE_2 anti-body were incubated together in capped eppendorf tubes for twenty minutes (9:1). A blank sample was prepared to represent zero analyte present. The test mixture was then pipetted ($100\mu\text{l}$) on the plate (replicated 5 times) and left on the plate for 15–20 minutes. They were then flicked out and washed with PBST three times before ABTS was added to the plate. The procedure is identical as the checkerboard titration from this point.

3.5.3 Commercial ELISA assay

A commercial kit for the detection of 17β Estradiol [Cat no 520 41] from IBL Immuno-Biological laboratories (Hamburg, Germany), assay procedure instructions given by the manufacture were followed as stated. Standard samples were prepared in PBS and synthetic wastewater. Real wastewater samples were analysed to determine level of Estradiol in the samples and then spiked real samples were used to validate recovery data.

3.5.4 Optimised assay for the Spreeta™ analyser

The gold surface of the sensor was cleaned prior to performing an assay with the regeneration buffer for 10 min and then washed for a further 10 min. This was important especially when using a new sensor after extended dry storage. Once washed the sensor was then dried with nitrogen gas stream prior to performing sensor initialisation. A typical procedure for performing an assay cycle was as follows:

- Before commencement of a experiment the sensor was initialised in air and the calibrated in water to establish a background reading were all following measurement would be referred to;
- PBS running buffer was flown past the surface for 5-10 minutes to obtain a baseline ($10\mu\text{g/min}$)
- $100\mu\text{l}$ of Estradiol BSA conjugate at an appropriate concentration was injected into the sample loop and then allowed to pass over the sensor surface after a PBS baseline was established.
- $900\mu\text{l}$ of sample was pre-incubated with $100\mu\text{l}$ of anti body $2.5\mu\text{g/ml}$ for.15-20 minutes.

- The proceeding sample solution was the injected into the sample loop and allowed to flow past the sensor surface at a rate of 10-60µl/ml for ten minutes.
- PBS again was the allowed to pass over the surface to remove any unbound antibody from the surface for a further 5 minutes to obtain a baseline measurement.
- The surface was then cleaned/ regenerated by flowing past regeneration buffer for 20 minutes followed by water for further 10 minutes.

Once the assay procedure was complete the cycle was repeated for the next sample measurement. Air initialisation and calibration in water was not required for subsequent measurements.

3.5.5 Immunoassay protocol for the Biacore® 3000

The immunoassay performed on the Biacore® 3000 as detailed below (*table 3.3*) is designed to resemble as close as possible to the assay performed on the Spreeta™ analyser. Therefore, J1 plain gold chips, filtered PBS buffer and Persil regeneration buffer were used.

Table 3.3 Immunoassay programmed assay protocol

Immunoassay Programmed Protocol (Biacore®3000)	
Running Buffer	PBS
Read point:	170 sec.
Water baseline:	Inject - 30 µl @180sec
Read point:	350 sec.
Running buffer:	Pass for 300 sec.
Read point:	530 sec.
E ₂ -BSA Conjugate:	Inject -100µl @600sec
Read point	1430 sec.
αE ₂ antibody	Inject 100µl@1450 sec
Read point	2050
Regeneration buffer:	Big inject 150µl @ 2100 sec
Read point	2930sec
Water wash & baseline:	Big inject 150µl @3300 sec.
Read point	3730 sec.
Running Buffer:	PBS
Read point:	4010 sec.

3.5.6 Data analysis used for the ELISA and SPR Immunoassay calibration curves

The amount of antibody binding in relation to the concentration of analyte present in the incubated samples for the ELISA assay was determined by subtracting the mean OD of the replicated analyte free wells (B_o) from the sample wells (B). Non specific binding (NSB) was accounted for by subtracting the mean OD of wells coated only with BSA (control wells). The formula used for the calibration graph is given below:

$$\frac{B}{B_o} = \frac{[B - NSB]}{[B_o - NSB]}$$

The ELISA calibration curves were plotted as B/B_o vs. $\text{Log}_{10} [E_2]$ and was fitted using a four parameter logistic concentration response equation:

$$y = \left[\frac{a - b}{1 + \left(\frac{x}{c}\right)^d} \right] + b$$

This was used to fit the concentration response curves to the data sets (*Sigma plot version 2.00, Jandel Corporation*). Where (a) is the maximum absorbance value, (b) is the minimum absorbance, (c) is the midpoint concentration producing 50% of the maximal absorbance and (d) is the slope at the inflection point of the sigmodal curve. The working range of the assay was defined by 10-90% of the B/B_o signal, the test midpoint is 50% of the B/B_o and the limit of detection was determined to be three times the standard deviation of the blank sample (*Mallat et al., 2001*). The SPR immunoassay data were analysed as detailed in *section 2.5.6*.

3.6 Results and discussion

The main objective of this chapter was to design and develop an immunoassay that could be easily transferred to a bio-sensing platform. The inhibition affinity assay has often been used for detecting environmental samples with optical sensors as it uses the heavy molecular weight of the antibody to be detected rather than the smaller lower weight molecules. This assay format has also been demonstrated to be very sensitive. With this in mind an ELISA plate assay was designed and optimised as an inhibition assay.

3.6.1 Development and optimisation of an EIA assay for waste water analysis

3.6.1.a The Design and Development of the ELISA assay

The combination of Estradiol-BSA coating and antibody dilution indicated in *Table 3.4*, which yielded an O.D of 0.8-1.0 at either 30 or 60 minutes after substrate addition in the checkerboard titration were considered likely to yield workable ELISA. Calibration curves were used to evaluate the performance of the indicated combinations. It was determined that the coating of conjugate at 50 μ g/ml gave the best coating converge. The decision criteria were whether the ELISA working range had an acceptable working performance at low Estradiol concentrations and minimum consumption of key reagents.

Table 3.4: Checkerboard titration assay schematic representation
Shaded combinations yielded an OD of 0.7 – 1.2 at ether 30 of 60 min. () combinations selected for further evaluation in the ELISA for Estradiol*

		17 β Estradiol BSA coating concentration (x2) (μ g/ml)								
		2	1	50	25	12.5	6.25	3.125	1.562	0.78125
Anti-body Concentration (μ g/ml)	1									
	0.5			*		*	*			
	0.25			*		*	*			
	0.125			*		*				
	0.0625			*						
	0.03125									

The shaded area of the table represents combination where the OD measurement between 0.7-1.2 at 30 of 60 minutes was seen. A time restriction of 60 minutes was used as to try to optimise the length of time of the assay. The stated (*) tested combination yielded suitable calibration curves. However, anti body concentration at 0.25µg/ml and coating at 50µg/ml was chosen due to the high constancy of results and low standard deviation of data points. It was noted that at low conjugate concentrations OD response was high but the curves produced from them were very erratic and displayed a high standard deviation.

3.6.1.b Optimised ELISA assay for buffered samples

Optimisation of the above chosen combination was required to give reliable results. Several parameters were investigated. Pre-incubation of the antibody and sample between 12 and 30 minutes was observed to be sufficient for the antibody/antigen interaction to reach equilibrium. At low incubation times the equilibrium was not reached and low binding was observed, at longer time intervals high OD response in antibody binding was observed. It was noted that if the pre incubated sample were left on the plate for more than 20 minutes a re-establishment of the test sample equilibrium (i.e. of the bound and unbound antibody in the solution) resulted in the antibody preferring the binding sites on the plate and therefore giving high OD levels across all the wells exhibiting a failed assay.

3.6.1.c Analysis results for spiked buffered samples using the developed ELISA assay

The figure below (*figure 3.2*) show a typical calibration curve given by the optimised developed ELISA.

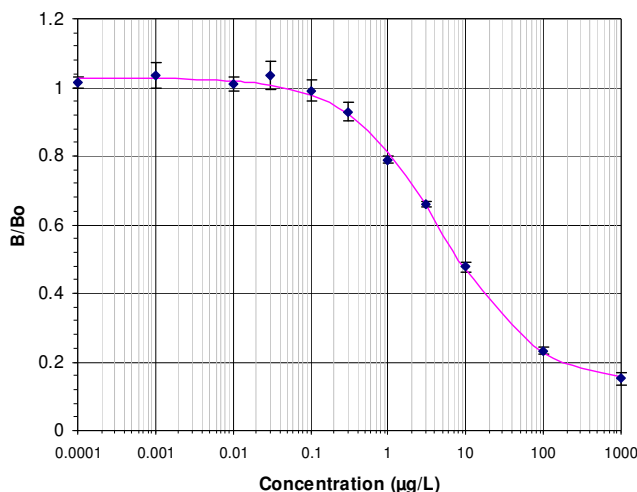


Figure 3.2: *The developed ELISA calibration curve for Estradiol in PBS pH 7.4 buffer. The above graph is a standard calibration curve obtained performing the developed inhibition immunoassay ELISA. The working range for this assay was between 0.3 and 100 µg/L. The curve has been fitted using a 4 parameter logfit. The error bars represent 1 standard deviation (n=5).*

The calibration curve for the developed ELISA (*please refer to figure 3.2*) had a working range of the assay was 0.3-70 µg/L with a limit of detection of 0.2µg/L. The test mid point of the assay was determined to be 10µg/L the assay is able to detect Estradiol in the sub pbb range and therefore could possible be sensitive enough for direct detection of Estradiol in real samples such as surface and waster water sample.

3.6.1.d Analysis of simulated wastewater samples

To determine how the assay behaved with wastewater. The calibration curve was performed this time with each calibrate made up in synthetic wastewater. One can immediately observer that the standard curve is much shallower but the working range would seam to have increased and the curve displays a more

linear shape. This could partly be due to matrix effect, but it is more likely to be due to sample buffering and the effect the ionic strength of the buffer has on the antibody. Although the sample was buffered using 5x PBS concentration increasing the concentration may suit the assay better. Collier et al 2002 mentioned the use of 1% ovalbumin introduced into the test sample. The rationale for this was that ovalbumin can act as an emolument and would protect the antibody and integrity of the protein structure.

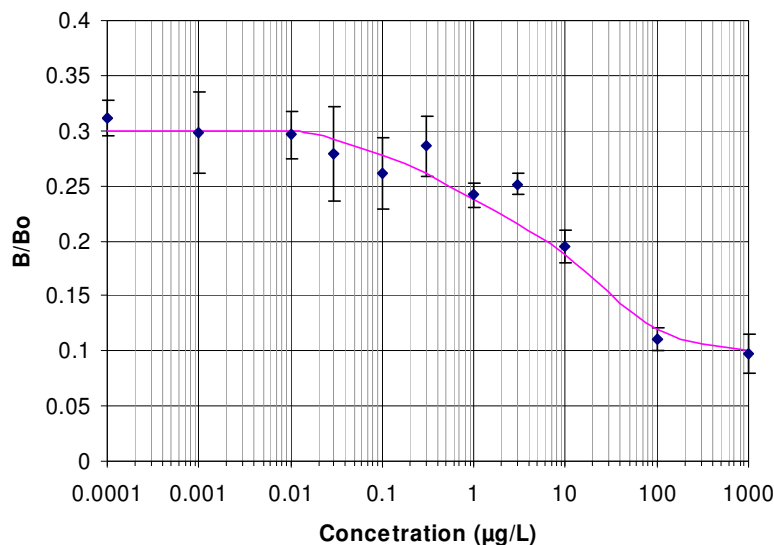


Figure 3.3: ELISA calibration curve for Estradiol in synthetic waste water. The above graph is a standard calibration curve obtained performing the developed inhibition immunoassay ELISA. The working range for this assay was between 0.03 and 100 µg/L. The curve has been fitted using a 4 parameter logfit. The error bars represent 1 standard deviation (n=5).

The assay performed in synthetic wastewater required further optimisation. It can be seen from the above figure (please refer to Figure 3.3) that the calibration curve is more linear than the curve with buffered samples and has an extended working range. The calibration curve had a working range between 0.1-100 µg/L and the lowest level of detection of 0.02 µg/L. Analyses of spiked synthetic wastewater were determined using the calibration curve for buffered samples. The sample was buffered using PBS (10x) and was corrected by finding the percent ratio of the blank sample in wastewater and that of the blank sample in the buffered sample.

3.6.1.e Analysis of Synthetic waste water samples and Real samples with micro titre plate-based assay

The developed ELISA was used to measure spiked samples of Estradiol in synthetic wastewater and real wastewater collected from Cranfield University sewage treatment works. Wastewater (A) was sampled from the influent and (B) was sampled at the effluent point.

Table 3.5: Analysis results of synthetic and real waste water samples using the developed ELISA assay

Analyte	True Concentration [$\mu\text{g/L}$]	Measured Concentration ($\mu\text{g/L}$)	Recovery Rate [%]
17 β Estradiol Synthetic waste water	0.3	0.35	117
	1	0.98	98
	3	3.35	106
17 β Estradiol Wastewater (A)	0.1	0.084	84
	1	1.13	113
	10	16.4	164
17 β Estradiol Waste water (B)	0.1	0.083	83
	1	1.15	115
	10	11.8	118

It can be seen from the above table (*table 3.5*) that the recovery rate was very good. According to the Association of Official Analytical Chemistry for Accuracy the recovery rates have to be in the range between 70-120% therefore, all but one meets this standard. The high value may have been due to a pipetting error. It was expected that the recovery rate for the spiked waste water sample would have been greater as there may have been Estradiol already present in the sample. The results indicate that this was not so or that it was present at such a low concentration it was unable to be detected using this method.

3.6.2 Development and Optimisation of Immunoassay on the field analyser

3.6.2.a Characterisation and optimisation of Estradiol assay on the analyser

Reducing the concentration of the antibody concentration decreased the change in sensor refractive index measurement during the incubation period. As the concentration decreases, the resulting binding curve resembles a straight line. At this point when the concentration of the antibody is sufficiently low, linear binding is achieved as antibody binding to the sensor surface becomes diffusion, rather than affinity limited. The curve for anti-E₂ antibody (0.25µg/ml) shows near linear behaviour. The sensorgrams has been normalised against the maximum value measured during each sample incubation period (*please refer to figure 3.4*).

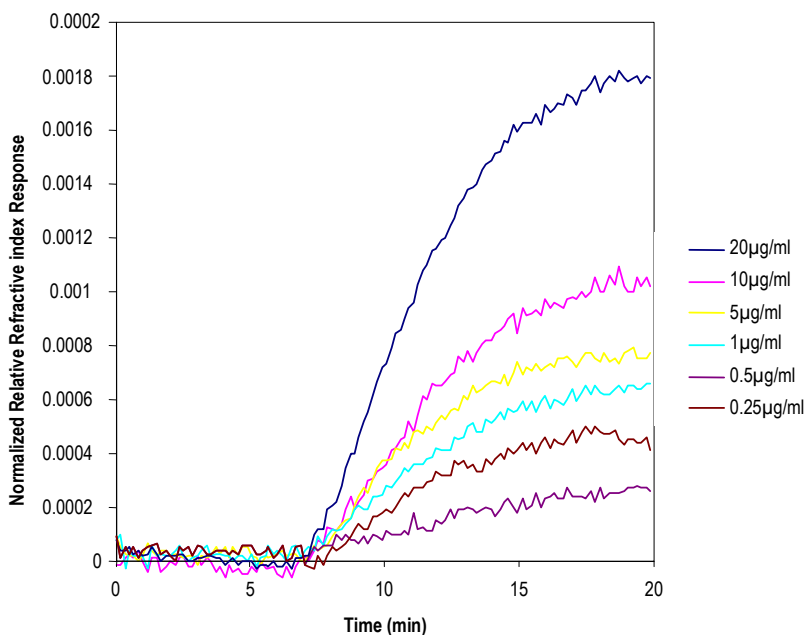


Figure 3.4 Sensor system characterisation examined by reducing the concentration of anti 17β Estradiol IgG antibody. A linear binding regime has been achieved with an antibody concentration of 0.25µg/ml. At this concentration the antibody binding to the sensor surface becomes diffusion limited, rather than affinity limited

3.6.2.b Sensor response to specific and non specific binding

Incubation of anti HCG to the sensing surface displayed very low non-specific binding, which was less than 1% increase in refractive index measurement of the PBS baseline (*please refer to figure 3.5*). This is approximately 3 times over the standard deviation of the PBS surface. Therefore very little non-specific binding had occurred at the sensor surface with HCG antibody (0.25 μ g/ml). Pre-incubation of the surface with 1% ovalbumin showed almost no refractive index change. This confirmed that the surface was well cover with absorbed protein and protected sufficiently from non-specific binding.

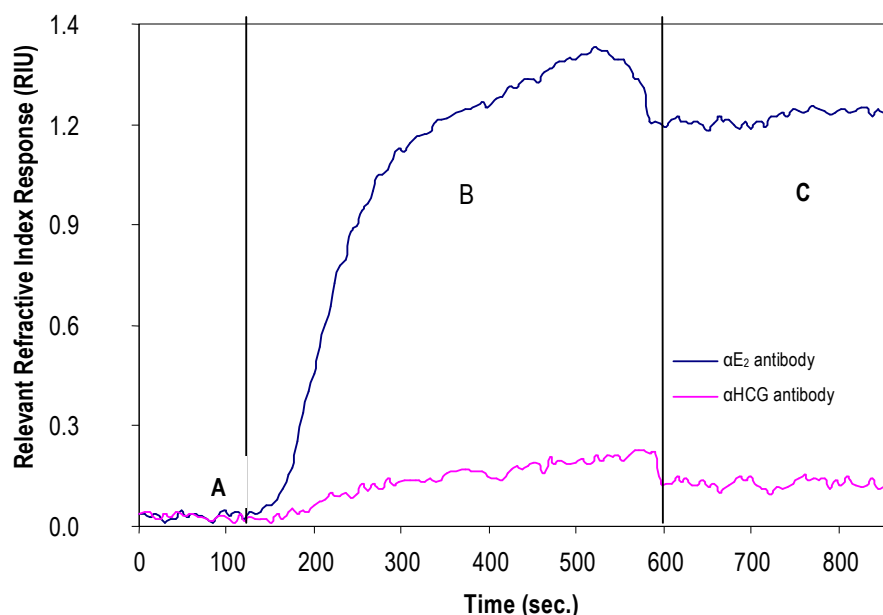


Figure 3.5 Response of specific antibody and non-specific antibody to the sensing surface. A) Represents the baseline for PBS. B) Incubation of antibody to the sensing surface and C) is the new PBS baseline. Where αE_2 anti-body shows specific binding to the surface and αHCG shows no binding to the physically absorbed biologically specific layer.

With BSA physical adsorbed on the sensing surface the anti 17 β Estradiol antibody was incubated on the surface to determine any non-specific binding of the antibody to a non specific surface. The non-specific base line time trace was subsequently subtracted from subsequent time traces. It was determined

that non-specific bind of the antibody was 97% lower, than the specific binding level.

3.6.2.c Analysis of buffered samples

A calibration curve for 17β Estradiol in PBS was performed. The assay took three days to complete a triplicate set of standards. Figure 3.6 shows one complete set of sample concentration measurements. Several issues concerning the procedure were addressed. Injection of high concentrations of analytes in the sample loop required extra cleaning with ethanol (90%) and PBST. This was done to make sure that there was no carry over of sample contaminating the next test sample.

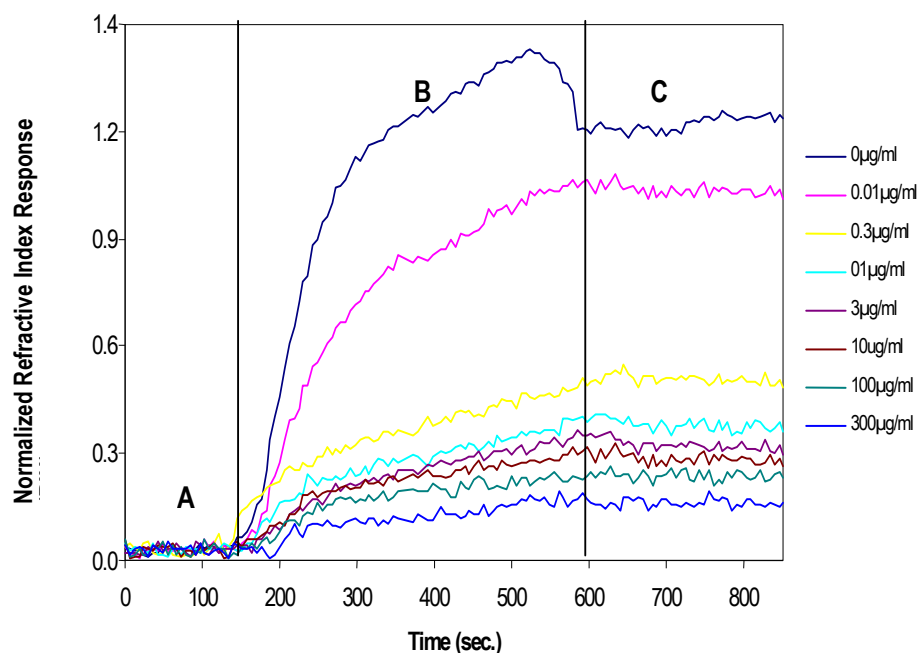


Figure 3.6: Typical real time SPR immunosensor response for several 17β Estradiol concentrations over the range of 0-300 μ g/L. Antibody concentration of (0.25 μ g /ml) A) Represents the baseline for PBS. B) Incubation of antibody to the sensing surface and C) is the new PBS baseline

The new 12 bit digital processor reduced the signal to noise ratio from 1.0×10^{-6} to 0.4×10^{-7} . This increased the sensitivity to refractive index change which was translated to the smoother sensorgrams.

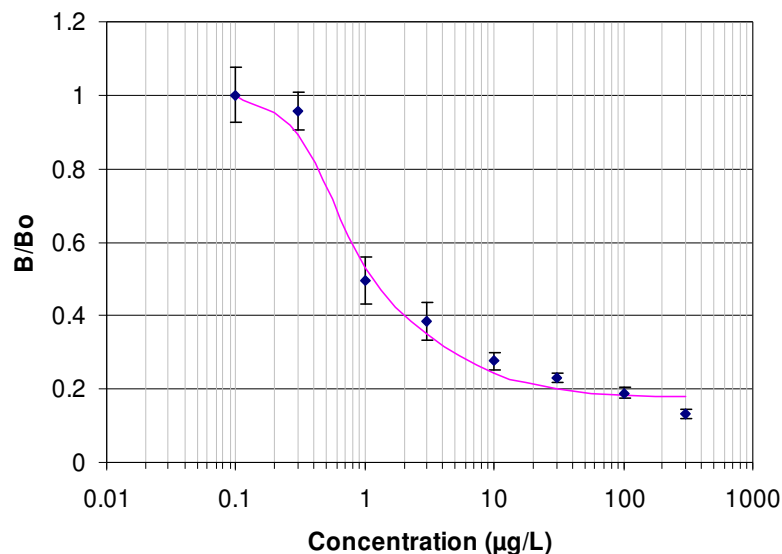


Figure 3.7: Calibration curve for Estradiol using the Spreeta™ analyser. The standard curve is derived from standard samples analysed in triplicate. The error bars indicate the standard deviation (1STD and $n=3$)

The calibration curve for Estradiol using the Spreeta™ analyser had a working range of 0.3- 7µg/ml with a lowest limit of detection of 0.2µg/l. The test mid point of the assay was determined to be 0.6µg/ml. The assay was able to detect Estradiol in sub pbb range and therefore could be sensitive enough for direct detection of Estradiol in real samples such as surface and waster water sample.

This assay showed very high sensitivity, which was comparable to the developed ELISA assay. It is often acknowledged that ELISAs are often one magnitude higher than that of a biosensor. As this is not true for this case this result may be due to the ELISA assay methodology. As the sample is continually being passed over the sensing surface the sample has no opportunity to preferentially bind to the sensing surface and therefore a true representation of the antibody and analyte inhibition equilibrium in the sample test pot can be monitored more accurately by the biosensor assay compared to the ELISA assay. However, the developed ELISA assay has a larger working range.

3.6.2.d Analysis of simulated wastewater sample

The assay for Estradiol on the analyser was conducted in synthetic wastewater. The calibration curve is given below.

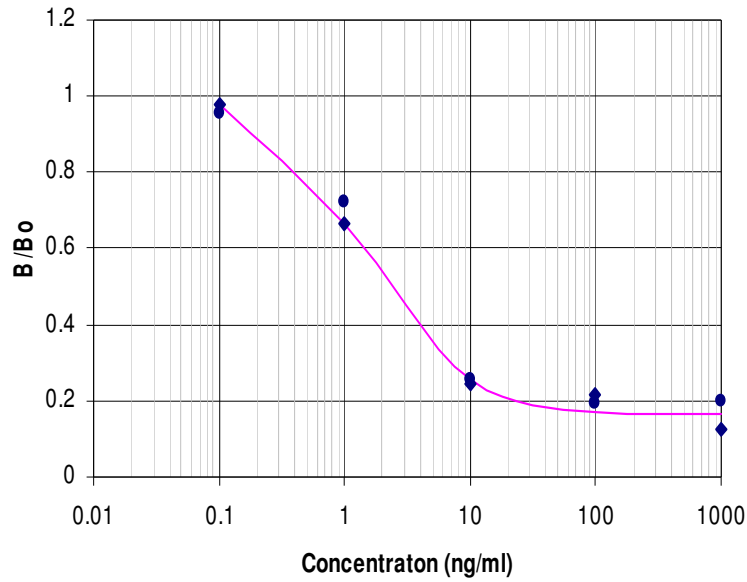


Figure 3.8: Calibration curve for β Estradiol in synthetic waste water using the Spreeta™ analyser. One data point represents a single analysis. Two data points are given for each concentration. ($n=2$).

The calibration curve for synthetic wastewater compared to the buffered sample curve shows a similar calibration curve to samples in buffered samples (please refer to figure 3.8). From the calibration graph the working range is approximately 0.1 to 10 μ g/L with a mid test point at 1 μ g/L. More data points would be required to give a more accurate calibrated working range. However, it can be observed that the data points obtained are very close together for each concentration sample.

Table 3.6 Analysis results of synthetic and real waste waster samples using the Spreeta™ analyser assay

Analyte	True Concentration [µg/L]	Measured Concentration (µg/L)	Recovery Rate [%]
17βEstradiol Synthetic waste water	0.3	0.28	93
	1	0.97	97
	3	3	100
17βEstradiol Wastewater (A)	0.3	0.29	97
	1	0.9	90
	3	2.25	75
17βEstradiol Waste water (B)	0.3	0.28	93
	1	1.2	120
	3	2.4	80

All of the recovery rates obtained by the Spreeta™ analyser was all within the acceptable range (*see table 3.6*). It was observed that matrix effect of the wastewaters did not have a significant effect on the obtained data. Buffered synthetic waster water and buffered real wastewater was used as the running buffer for the analysis. This may have reduced the matrix effect by priming the surface prior to the sample being introduced to the surface. Filtering the wastewater may also have been sufficient enough to reduce any possible matrix effect. It was also noted during the course of investigation that matrix effect did occur, represented by increased refractive index when synthetic wastewater that was over a week old was used. This was considered to be due to microbial growth in the buffer system.

3.6.3 Immunoassay of 17β Estradiol on the Biacore® 3000

3.6.3.a Analysis of buffered samples

The same assay that was designed for the analyser was transferred directly to the Biacore® 3000 system. The only difference being that the assay cycle was automated, which allowed for high throughput of replication for each sample calibrate.

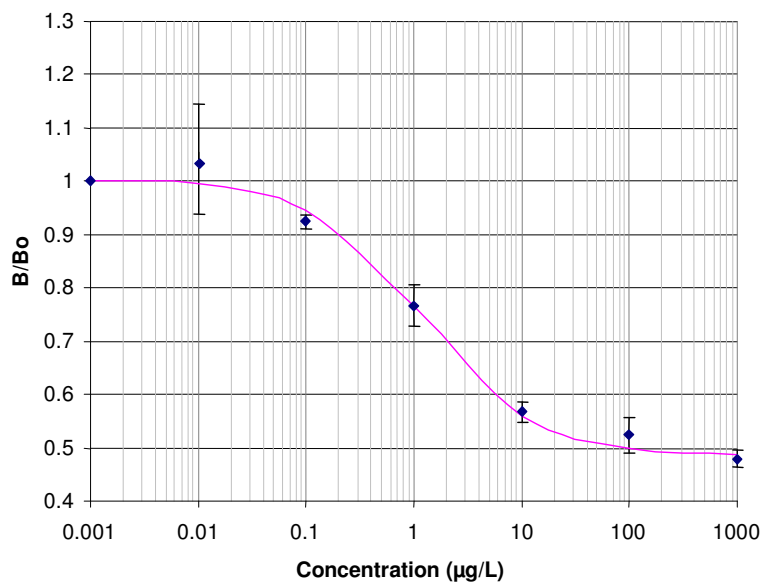


Figure 3.9: Calibration curve for Estradiol using the Biacore® 3000. The standard curve is derived from standard samples analysed in triplicate. The error bars indicate the standard deviation (1STD and $n=3$)

The calibration curve for Estradiol using the Biacore® 3000 (please refer to figure 3.9) shows a working range of 0.1- 10µg/L with a limit of detection of 0.1µg/L. The test mid point of the assay was determined to be 1.0 µg/L. The assay was able to detect Estradiol in sub pbb range and had a working range similar to that of the Spreeta™ analyser. Therefore, could be sensitive enough for direct detection of Estradiol in real samples such as surface and wastewater samples.

3.6.3.b Analysis of simulated wastewater samples

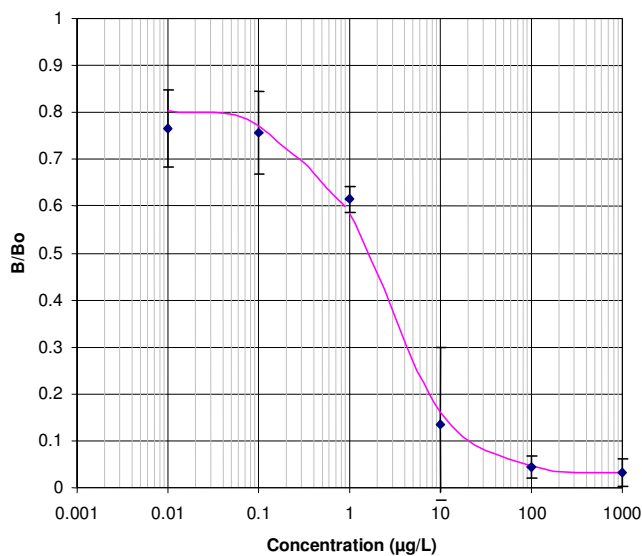


Figure 3.10: Calibration curve for 17β Estradiol in synthetic waste water using the Biacore® 3000 analyser. The standard curve is derived from mean of three samples of each concentration. The error bars indicate the standard deviation (1STD and n=3)

For the calibration given for synthetic wastewater using the Biacore ®3000 (please refer to figure 3.10), the working range of the curve is more pronounced than the one for the buffered samples. The working range of the assay was 0.1- 10µg/L with a limit of detection of 0.1µg/L. the test mid point of the assay was determined to be 2.0µg/L. The working range of the two calibration curves for buffered and synthetic waste waster was very similar.

Table 3.7: Analysis results of synthetic and real waste water samples using the Biacore®3000 assay

Analyte	True Concentration [µg/L]	Measured Concentration (µg/L)	Recovery Rate [%]
17β Estradiol Synthetic waste water	0.3	0.34	113
	1	1.0	100
	3	2.9	97
17β Estradiol Wastewater (A)	0.3	0.20	67
	1	0.90	89
	3	2.7	90
17β Estradiol Wastewater (B)	0.3	0.26	87
	1	0.95	95
	3	2.8	93

The Biacore® 3000 recovery rates as shown in the above table (*please refer to table 3.7*) were all within the acceptable range and were very close to the true concentration of the test samples when determining them from the given calibration curves.

3.6.4 Determination and validation of EIA and Immunoassay Result using a commercial ELSIA Kit for immunoassay

A commercial ELSIA test kit was used to determine the level of 17β Estradiol in real water samples and to compare the performance of the other developed assays.

3.6.4.a Analysis of buffered samples

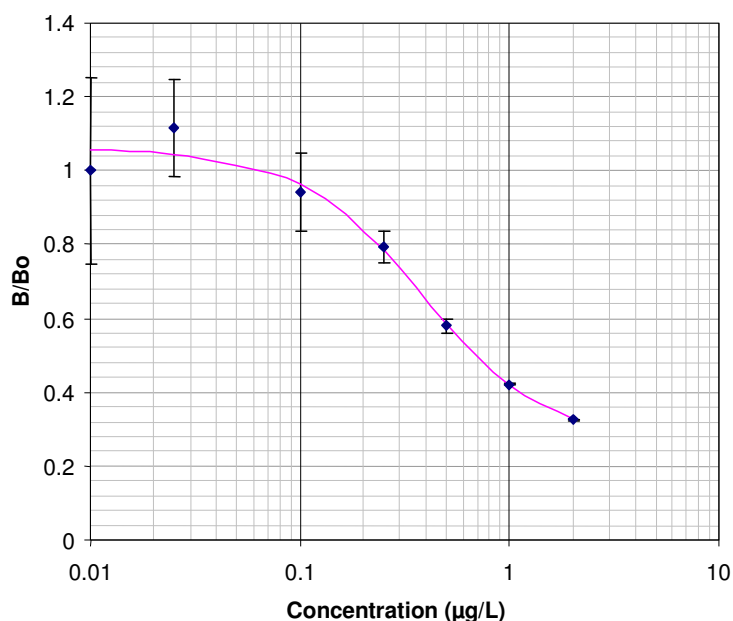


Figure 3.11: Calibration curve for Estradiol using IBL Estradiol ELISA kit. The standard curve is derived from standard samples analysed from the mean of five wells. The error bars indicate the standard deviation (1STD and n=5)

The working range of the assay was 0.1- 2 µg/L with a limit of detection of 0.05µg/L. The test mid point of the assay was determined to be 0.3µg/L (*please refer to figure 3.11*). The assay would be sensitive enough to detect Estradiol in the sub-ppb range and had a working range that was smaller, but marginally better due to being able to detect Estradiol at lower concentrations than the other assays. The IBL calibration assay is normally calibrated using serum medium. It was noted that the standard curve for buffered samples was shallower than the serum calibration curve. However, each buffered concentration sample showed much tighter standard deviation than that

obtained by the serum curve (data not shown). The assay should be sensitive enough for measuring Estradiol in real environmental sample

3.6.4.b Analysis result for spiked synthetic wastewater samples

The calibration curve for synthetic wastewater samples shows a near linear line with a large working range. The calibration working range is perceivably extended.

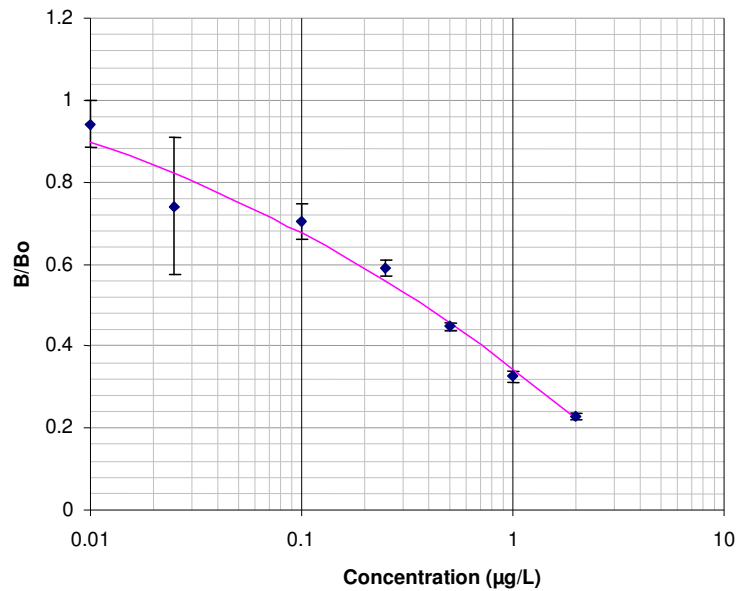


Figure 3.12: Calibration curve for β Estradiol in synthetic wastewater using the IBL ELISA Kit. The standard curve is derived from mean of five samples of each concentration. The error bars indicate the standard deviation (1STD and n=5)

The calibration curve (*figure 3.12*) shows a linear concentration range from 0.01-2µg/L could be used to calculate for concentrations. To analysis the working range assay limits for this calibration curve more data points increasing the range would be required.

Table 3.8: Analysis results of synthetic and real waste waster samples using the IBL ELISA kit assay

Analyte	True Concentration [$\mu\text{g/L}$]	Measured Concentration ($\mu\text{g/L}$)	Recovery Rate [%]
17 β Estradiol Synthetic waste water	0.1	0.09	90
	0.3	0.26	78
	1	0.92	92
17 β Estradiol Wastewater (A)	0.1	0.1	100
	0.3	0.34	119
	1	1.1	110
17 β Estradiol Waste water (B)	0.1	0.09	83
	0.3	0.96	74
	1	0.88	88

The measured spiked samples in synthetic wastewater and real wastewater all fell within the acceptable recovery range limit (*please refer to table3.8*). Blank samples of just real wastewater from both sites were assayed to determine whether there was any residing Estradiol present in the collected sample prior to it being used for spiked test sample analysis. The result obtained indicated that there was no to very low concentration of Estradiol that could be detected using the commercial ELISA test kit.

3.6.4.c Summary of analysis samples in pure buffer, synthetic waster water and real samples

For all of the samples measured in synthetic wastewater and real wastewater samples only one of them fell out of the accuracy range. The working range for the developed ELISA was (0.3-70 μ g/L), for the Spreeta™ Analyser (0.3-10 μ g/L) and for Biacore® 3000 (0.1-10 μ g/L). All of them show similar working ranges and were able to detect spiked samples of Estradiol in synthetic and real wastewater samples with very little sample pre-treatment.

3.6.5 Design of the Proposed automated liquid handling system

During the course of the research into developing a SPR based analyser. It was obvious that automation of the fluidics would be an ideal situation. A design for the automated liquid handling system was proposed and is feature below. The proposed design incorporated valves and a piston pump from Lee Electronics (*Bucks. UK*). These components were chose as they were miniaturised and the whole system would be less than an A4 sized page. Due to time and monetary constraints the fluid handling system was unable to be realised. It was hoped that automation of the assay protocol would aid further assay development, increase sample reproducibly and demonstrate real remote sensing and surface regeneration in the field.

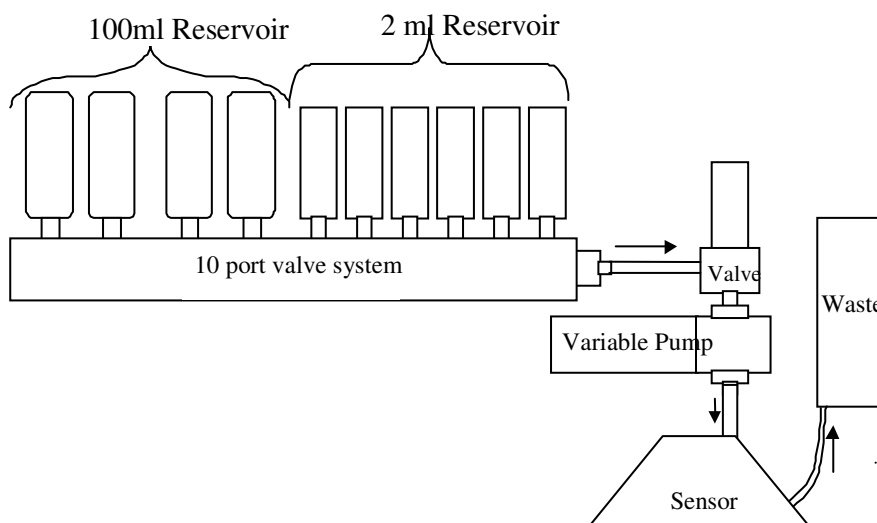


Figure 3.13: Schematic design of the proposed miniature liquid handling system for the Spreeta™ field analyser. All of the components were to be from Lee Electronics,(Bucks UK)

3.7 Further Discussions and Conclusions

Surface plasmon resonance as a bio sensing technique is a very power tool, as it requires no labelling. The Spreeta™ sensor is very robust and over the years of development it has improved on sensitivity and reliability. In this chapter it has been demonstrated that the sensor can be used quantitatively as a biosensor with detection limits as low as 0.1µg/L.

The developed ELISA assay showed good sensitivity with a working range of 0.1-10µg/L. However, it was observed that by leaving the pre-incubated test sample on the plate for more than 20 minuets had an affect on the sample in solution equilibrium where the antibodies bound more readily to the plate surface than the free antigen. A competition assay format with the antibody and analyte incubated together on the plate did not work either. In a paper by Sherry et al. (*Sherry et al., 1999*) the authors also noted this similar phenomenon. They had vitellogenin coated on their plates and their polyclonal antibodies preferentially bound to the plate surface.

According to the Association of Official Analytical Chemistry for Accuracy the recovery rates have to be within the range of 70-120%. For all of the samples measured only one fell out of this range. The real wastewater samples were taken from Cranfield University sewage treatment works. It was expected that Estradiol would have been detected in the blank samples. However, results of the blanks using the commercial ELISA kit showed no detectable level. The population of males to females on the campus is approximately 8:2. Therefore, it is not surprising that the level of Estradiol was not high enough to be detected by the ELISA kit. A sample extraction technique and HPLC analysis may have been able to confirm this.

4 Photo Modulation of bioactive proteins for sensor regeneration applications

Chapter 4

Photo Modulation of bioactive proteins for sensor regeneration applications

4.1 Introduction

The major advantage of using immuno-sensing techniques for detecting low molecular weight analytes is the use of high affinity antibodies. The high affinity binding of the antigen-antibody complex on one hand allow the sensor to be a very effective biosensor as it would be intrinsically highly selective and highly specific to its target analyte. However, this strong association more often than not makes it very difficult to achieve complete dissociation and regeneration of the sensing surface after the sensing binding event (*Willner et al., 2001*) . Hence, most developed biosensors that utilize immuno-affinity molecules are single use devices.

The development of an effective reversible and therefore reusable immunosensors would certainly be a major advance in this field, especially when applied to environmental pollution monitoring and remote sensing where sensors could be placed in environments that are either difficult or too hazardous to interfere with, and true continuous monitoring could be achieved where continuous measurements of analytes is required (i.e. in underground oil tank and aquifers, and underground water treatment pumps).

Photo-chromic molecules are compounds that are able to exist in different isomeric forms that are very different from each other in geometry and chemical properties; they can change their form by being exposed to different wavelength illumination ranges switching from visible [400-700nm] to ultra violet [200-390 nm]. This unique conformational chemical property, these compounds have could therefore be utilised as molecular switches when applied to bioactive systems. The incorporation of these photo-stimulated molecules to bioactive proteins could in theory interact selectively with the proteins bio-activity. Therefore, realising the possibility of true reagent-less and reversible biological activity and therefore advancing the applications for biosensors.

This chapter will explore and characterise the behaviour and mechanism of a particular photochemical dye belonging to the spiropyran group of compounds. The photo-chromic dyes via covalent attachment will be coupled to the backbone frame of selected bioactive proteins. The photo-modulation of these bioactive proteins due to dye coupling will be assessed and its feasibility to integrate photo-modulation in an immunoassay system for regeneration applications is investigated.

4.1.1 Sensor regeneration applications approaches

For the most part, commercially developed biosensors utilise cheap “single-cycled” sensor chips or electrode arrays with bioactive surfaces that can be decoupled from the sensor transducer electronics that is necessary to produce the detection signal. In cases where these surfaces are regenerated for repeated use there is often a loss in sensitivity due to incomplete dissociation of the binding recognition molecule to its analyte and/or damage (e.g. irreversible denaturing) to the biological surface active layer caused by the regeneration buffer used to decouple the affinity complex. These buffers are often unpleasant and very aggressive by nature as they are used to disrupt naturally

occurring binding events. To date, different regeneration strategies have been used the most commonly used are extreme pH buffers (e.g. glycine-HCL-buffer, NaOH and Triton 100 X buffers at pH 2 or 13 respectively), high concentration of surfactants such a SDS, saturated salt solutions such as urea, concentrated solvents e.g. >85% ethanol and chaotropic reagents such as proteases (e.g. pepsin and/or papain) (*Andersson et al., 1999; Wijesuriya, 1994*). For some of these regeneration buffer examples given above can completely regenerate some antigen-antibody complexes depending on their affinities. However, more often analysis allowances are giving to the drop in surface sensitivity and often in systems such as optical detection systems the sensor surface requires the first batch of result to be discarded until the sensor surface has been used several times (often referred to as surface conditioning) to get a relatively stable platform. However, in all these cases regeneration requires the introduction of reagents that affects the binding complex therefore continuous measurements is not achieved.

By integrating photo-responsive molecules to bio-affinity system, regeneration of the sensing surface after a binding event could be achieved by the illumination of the surface at an appropriate wavelength and then simply washing away the dissociated affinity partner protein with the running buffer. Rapid reversible modulation of bio-affinity would provide continuous, real-time monitoring. This would offer the convenience of a reagent-free, rapid, ligand release system (*Kirkham, 1996*).

4.1.2 Photo-chromisium and Spiropyran Dyes

Substances that undergo reversible colour formation under light irradiation are called photo-chromic compounds (*Karube et al., 1976*). Spiropyran molecules are bicyclic compounds with one and only one atom that is common to both rings. The photo-chromic spiropyran molecules are composed of two π -electron moieties that are orientated orthogonal to each other. The configuration of the compound means that each part exhibits its own individual absorption spectra rather than that of a complete conjugated system. However, when illuminated with an appropriated light sources (e.g. electromagnetic excitation) bond cleavage takes place and the molecule reconfigures its self into a near planar structure referred to as the merocyanine form. The π -electron system is now extended throughout the molecule and a bathochromic shift from *U.V.* absorption region to the visible region occurs (also know as a red-shift in the absorption spectrum) in the open merocyanine (*Martinek et al., 1978*) (*see figure 4.1*).

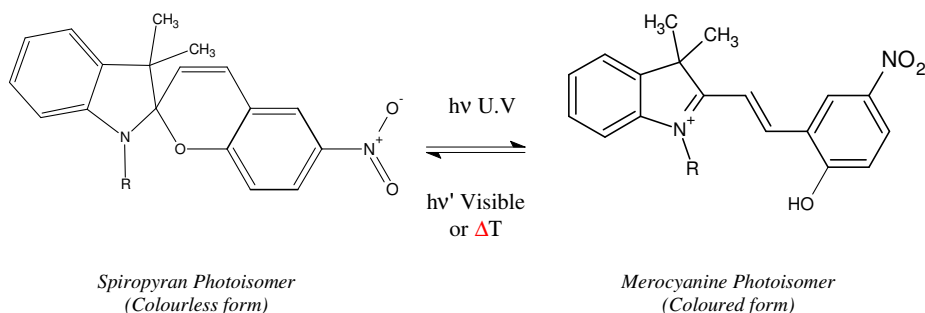


Figure 4.1: Schematic of the photo-isomerisation of a generic spiropyran compound. The spiropyran photo-isomer has a neutrally charged and is colourless in non-polar solvents. Illumination under UV light converts the spiropyran photoisomer into the coloured merocyanine form. The merocyanine photoisomer is a highly conjugated compound, giving a red coloured solution. Upon visible light illumination of the merocyanine form converts spontaneously to the spiropyran form and with the removal of UV illumination a slow dark conversion to the spiropyran colourless form also occurs.

In non polar solvents spiropyran compounds 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 highly coloured (i.e. which gives it its dye classification) and a zwitterionic compound and therefore more hydrophilic. Hence, the spiropyran dye not only has the ability to change the geometry of its configuration and absorption profile but also each form has a drastically different polarity. It is for this reason why spiropyran dye have been of interest in the scientific world and why in this course of investigation was chosen as the preferred photochromic dye over others that have only a geometrical change in their isomeric forms (e.g. azo dyes and fulgidides).

4.1.3 Photo-modulated control of biological systems

Photosensitive molecules occur naturally and have had a great impact in animal and plant evolution. Naturally occurring photoreceptor usually contain low-molecular weight photo chromic molecules that are bound to a macromolecular matrixes (e.g. membrane proteins). On irradiation the photo-chromic moieties undergo reversible stoichiometric rearrangements, which in turn induces structural changes in the macromolecular matrix (*Pieroni et al., 1992*). Photo-chromic compounds are ubiquitous in nature, they exhibit themselves as physiological molecular triggers for highly important photo-regulated biological processes, and two great examples are:

1. **Vision in animals** is controlled by a photosensitive molecule rhodopsin, which is attached via a lysine residue group on the retinal membrane. Upon exposure to light a conformational change occurs in the molecule from a “*cis*” isomer to an all “*trans*” isomer. This change is then accompanied by a series of cascading dark events due to further conformational induced changes in the molecular matrix causing variations in the optical membrane permeability, which eventually leads to neural impulses and visual perception (*Pieroni et al., 1992*).
2. **Photosynthesis in plants** is controlled by phytochromes, which in turn control several morphological and developmental responses. Phytochrome is a chromo-protein that contains a linear tetrapyrrole chromophore as a prosthetic group. Illumination leads to a conformational isomerisation reaction of one or more the conjugated bonds of the tetrapyrrol molecule. This initial event induces a reversible α -helical folding of the protein (*Roberts, 1986*). This response is induced by red light (660nm) and inhibited by far-red light (730nm). The photo-chromic behaviour of the molecule leads to the photo-reversibility of the signal. Hence, photosynthesis in the day during daylight and respirations at night.

Three observations can be extracted from the above examples that demonstrate the properties naturally occurring photo-sensitise biological systems have (1) their macro-molecular matrixes (protein membranes) have low molecular weight photo-chromic moieties covalently attached into their structure. (2) Upon external irradiation with appropriate wavelength reversible stereo-chemical rearrangement between one or more isomeric forms occur, (3) initial photochemical reaction induces conformational changes in the macromolecular structure causing direct or indirect physiological photo-responsive effect (*Pieroni, 1992*).

Photo-modulation of bioactive proteins by the introduction of synthetic photo chromic molecules have been investigated and successfully accomplished. Willner *et al.* (Willner *et al.*, 1994) demonstrated that by covalently attaching thiophenylfulgide active ester onto the protein backbone of α -chymotrypsin they were able to reversibly control its bio-catalytic activity in organic solvent upon appropriate illumination. Song *et al.* (Song *et al.*, 1995) showed that by introducing azobenzene moieties into phospholipids they were able to demonstrate photo-reactive super molecular assemblies. That is photo induces bi-layers and aggregation. This highlights that by combining azobenzene or related compounds with natural phospholipids, the construction of synthetic photo-regulated membranes and related materials is a possibility. Azo dyes have also been used to photo modulate the activity of the enzyme azoaldolase and papain (Willner *et al.*, 1990) as well as a photo-reversible inhibitor of cysteine and serine proteases (Westmark *et al.*, 1993).

4.1.4 Bio-modulation using photosensitive Spiropyran dyes.

Spiropyran derivatives are an important class of photo-and thermo chromic compound that can be easily converted to the zwitterionic merocyanine isomer. The isomerization is unique in terms of accompanying large changes in structural and electrical characteristics of the molecule (Inouye, 1996). Spiropyran dyes have been used to modulate biological proteins. Most of the research has looked at the incorporation of spiropyran dyes attached to relatively small molecules. With the conceptual idea that by covalently attaching reversible photo- isomerization compounds to bioactive molecules (e.g. Concanavalin A or HRP), in one photo-isomerizable state of the photo-chemical unit, the protein attains its tertiary bioactive structure and is in the “on” state, while photo-isomerization to the complementary state results in a deactivation of its biological function effectively switching “off” the bioactive protein (*please refer to figure 4.2*) (Zahavy *et al.*, 1994; Willner *et al.*, 1993).

This model has proved to be successful, Kaganer *et al.* 1999 showed a reversible immunoassay for Dinitrophenol (DNP) using surface plasmon resonance, (Kagner *et al.*, 1999). However, in this example photo-modulation was demonstrated via the photo modulation of the antigen and its affinity to its antibody rather than the antibody affinity to its antigen.

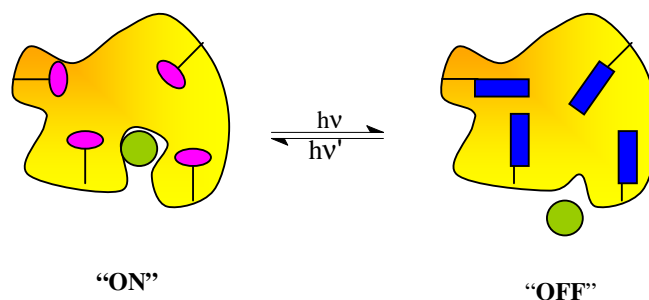


Figure 4.2: Schematic representation of the protein backbone modified by photo chromic groups. The different photo-isomerizable forms changed by appropriate illumination affect the activity of the protein. In the “on” state the protein structure is unaffected by the covalently attached photo-isomer and therefore normal bioactivity is allowed. In the off state the introduced photo-isomer has switched to its other form by being exposed to an external illumination and this in turn affect the bio-activity of the protein, effectively turning the bioactive protein off.

It is therefore possible that by modifying the protein structure of an antibody with photo-chromic moieties rather than the antigens, photo-isomerisation switching may also induce a change in the affinity of the antibody complex to its antigen. This scheme would also prove to be an effective and flexible way of establishing a renewable biosensor platform for use in remote situations, especially for optical sensors and the detection of low molecular weight pollutants.

4.2 Aims

The aim of the work in the following chapter was to synthesise a well characterised form of a spiropyran dye (*1-(b-carboxyethyl)-3,3-dimethylnitrospiro [indoline-2,2'-22-H-benzopyran]*). The intention was to utilize the dyes unique features and attempt to attach the dye to bioactive proteins to evaluate the possible bio modulation of the bioactive proteins in solution and on solid supports. The objective of the study was to develop a reagent-less bio-reversible sensing surface for immunoassay applications that could be utilised in a remote or portable field analyser. To realise this horseradish peroxidase was used as a model bioactive enzyme and two monoclonal antibodies: anti-fluorescein and anti 17β Estradiol was used.

4.3 Materials and Methods

4.3.1 Chemicals, biological compounds and equipment

3-iodopropionic acid (Sigma code: I-1, 045-7); 2,3,3-trimethylindolenine (Sigma code: T-7, 680-5), Ethanol (Sigma Code: BCR656), Toluene (Sigma code:590835), Methyl Ethyl Ketone (MEK) (Sigma code T-4428), piperidine (Sigma code P5881), 2-nitrosalicylaldehyde (Sigma code N-5280), Methanol (Sigma code.M1770), horseradish peroxidase (Sigma code P-6782), Anti-FITC monoclonal antibody (sigma code: F5636), FITC-HRP (sigma code:P-2649), ABTS (code: A-9941sigma), SiO₂ TLC plates, IR cuvette, visible light source and UV lamp. Anti- 17 β Estradiol, 6 Estradiol –6-CMO-HRP [cat: 65-IE16] (Fitzgerald Industries International inc., MA, USA). UV light 245 nm, Ultra violet light source UVG-54, UV products ltd, San Gabriel, CA: 245nm, 0.12 amps at 220 volts), daylight filament bulb, 50W (Philips).

4.3.2 Spiropyran dye synthesis and characterisation

4.3.2.a Chemical synthesis of 1- (β -carboxyethyl)-2,3,3-trimethylindolenium iodide: Precursor for the production of a carboxylated spiropyran.

For the synthesis of the carboxylated spiropyran, the compound β -iodopropionic acid was required to derivatise 2,3,3-trimethylindolenine to form the carboxylated precursor compound 1-carboxymethyl-2,3,3-trimethylindolenium iodine. The dye was synthesised using a modified method (Kirkham, 1996) adaptation of Aizawa, Namba and Suzuki (Aizwa *et al.*, 1977) method (*see figure 4.3*).

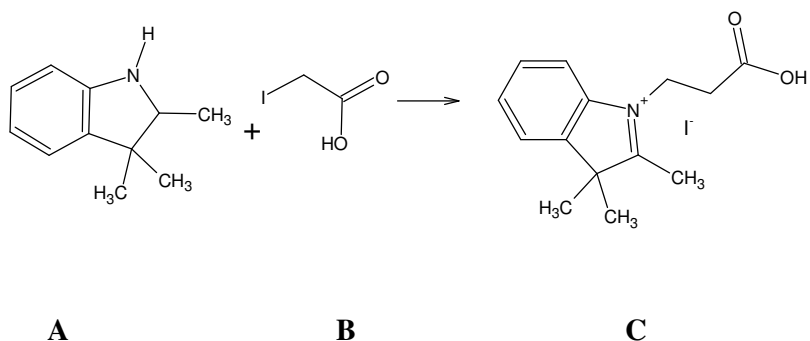


Figure 4.3: A reaction schematic of the synthesis of **1-carboxyethyl-2,3,3-trimethylindolenium Iodide**. Where (A) 2,3,3-trimethylindolenine, (B): 3-Iodopropionic acid and (C): 1-carboxyethyl-2,3,3-dimethylindolenium iodide. In equimolar amounts both compounds are refluxed together at 80°C for three hours then a further 1 hour at 100°C devolved in 20% v/v ethanol in toluene. The mixture is allowed to crystallise over night at room temperature

An equimolar mixture of 3-iodopropionic acid (7.55g) and 2,3,3-trimethylindolenium iodide (6.00ml) was heated at 80°C under reflux for 3 hours and then 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 1 hour and left overnight at room temperature. The purple precipitate was collected by filtration, and the solution was retained for further crystallisation. The purple solid was crushed using a glass pestle and mortar then washed with 5% ethanol in toluene. The filtrate was retained for further crystallisation.

In order to gain a pure substance, the solid obtained was refluxed at 100°C in fresh toluene, using sufficient ethanol solvent to dissolve all the material (approx. 5-7%). This was left overnight at 4°C to re-crystallise. The crystals were filtered off and dried to give a yellow crystalline product. This was further filtered and 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 was filtered and was stored in the dark at room temperature.

4.3.2.b Synthesis of Carboxylated Spiropyran

The method used to synthesise the spiropyran was based on a modified method of Namba and Suzuki (*Namba et al., 1975*) (see figure 4.4).

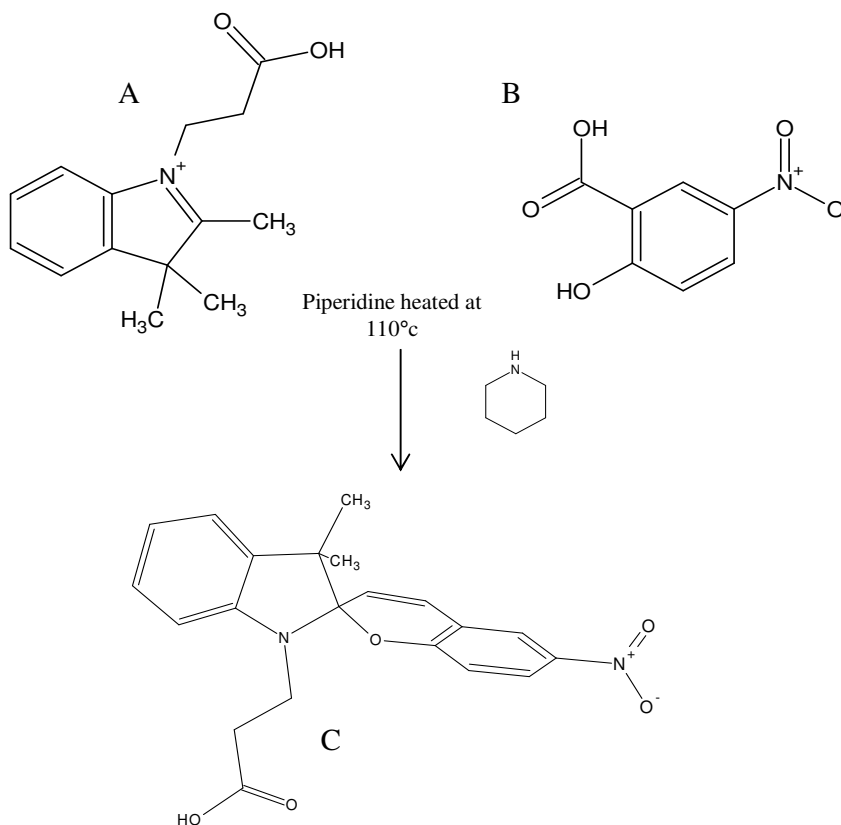


Figure 4.4: Schematic reaction sequence to synthesis the spiropyran dye. A: 1-carboxymethyl-2,3,3-trimethylindolenium iodide, B: 5-nitrosalicylaldehyde and C: 1-β-carboxyethyl)-3,3-dimethylnitrospiro[indoline-2,2'-22-H-benzopyran

The quaternary ammonium salt product of the first reaction: 1-carboxymethyl-2,3,3-trimethylindolenium iodide (*section 4.2.2,a*) was suspended in methyl ethyl ketone (MEK)[sigma code: M2886] (500 mg of solid in 600μl of MEK) in a round, flask, The suspension was dissolved by the addition of piperidine (125 μl) and heated under reflux at 110 °C until all solid was in solution

The reaction mixture was taken off the heat and 2-nitrosalicylaldehyde (250 mg) in 2ml of MEK was added to the reaction mixture and 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 was filtered and washed under a vacuum with 50 ml of water and left to dry. The resultant solid was stored in the dark at room temperature.

4.3.2.c TLC analysis of spiropyran dye

To ascertain the relative purity of the synthesised crystalline end compound, thin layer chromatography using Silica oxide plates and aluminium oxide plate (Aluminium oxide 60 F₂₅₄, neutral type E, Merk code A5581) was performed using 99.9% ethanol as the mobile liquid phase. A 1mg/ml solution of the crystalline end product was made in pure ethanol and 2.5 µl of the solution were spotted onto a TLC plate. All of the reaction compounds used for the dye synthesis were also spotted at the same concentration (1mg/ml) and used to compare for reaction identification. The resulting positions of compounds were viewed in ambient light (i.e. silica oxide plate) and under UV light [245nm] (for aluminium oxide plates) and the positions measured, The R_f values were determined as the distance of the spots mid-point divided by the solvent front in millimetres. Therefore, all R_f values are equal or less than 1.

4.3.2.d Photo chromic activity of SP-COOH in different solvent buffers

To determine and demonstrate the successful synthesis of the carboxylated spiropyran and the product's photo-chromic activity, a series of experiments were performed. A 1mg/ml solution of the spiropyran dye (SP) was made using ethanol (99.9%). Of this solution 100µl was taken and made up to 100ml in the ethanol (99.9%), methanol (99%) and MES (10mM, pH 6.7) buffer.

Taking 1.5ml aliquot of each of the SP solutions using appropriate solvents as blanks, was placed in individual quartz cuvettes (path length: 1cm) and assayed using a spectrophotometer (M350 UV visible spectrometer). Each solution was scanned between 200 and 650nm and zeroed at 800nm after being stored of 24 hours in the dark at room temperature; this was used as the baseline profile of photo-chromic activity due to dark adaptation. Then each cuvette solution was illuminated with UV light (*UVG-54, UV products Ltd, San Gabriel, CA. [Peak output: 245 nm, 0.12 amps at 220 volts]*). The solution was exposed to the light for 20 minutes scanned and then illuminated for a further 20 minutes in visible light (*daylight filament bulb, 50W Phillips*) and scanned. The light source was placed 10 cm away from each cuvette.

To determine the natural fade back of the merocyanine form to the spiropyran form or vice versa a 1 ml aliquot of 1mg/ml solution was illuminated for 15 minute with UV light the sample was then scanned every two minutes until the absorption profile stabilised.

4.3.3 Attachment of the spiropyran dye to bioactive protein modulation studies

4.3.3.a Conjugation of spiropyran dye to bioactive proteins

To conjugate the spiropyran dye to the protein of interest (i.e. Horseradish Peroxidase and Anti-FITC monoclonal IgG), 1 mg of dye was dissolved in 0.25 ml of 100% ethanol and then made up 4ml in 0.1 M MES, (pH 6,8). A protein solution of 2 mg/ml was made up in 0.1 M MES (pH 6.8). The protein solutions of interest and the dye solution were mixed together. Then 30 mg of 1-ethyl-3 (3-dimethylaminopropyl) carbodiimide (EDAC) and 15 mg NHS were added to the solution and stirred until dissolved. The reaction mixture was allowed to incubate for up to 4 hours, with gentle mixing at room temperature (*Weston et al., 1999a; Hermanson et al., 1992*).

4.3.3.b Spiropyran conjugated protein purification

The resultant reaction mixture (section 4.3.3.a) was purified using a Sephadex PD10 column (*Pharmacia, Uppsala, Sweden. Exclusions limit 5×10^3 MW, code: 17-0851-01*) pre- equilibrated with 25 ml of 25% ethanol in 0.1 M MES, pH 6.8. A 2.5ml aliquot of the dye-protein reaction mixture was pipetted into a PD 10 column. Once the sample had entered into the column it was followed by 3.5ml of eluting buffer. Eluted 1 ml sample fractions were collected and carefully numbered in order of elution. The fractions that passed through the PD10 column were assayed for dye attachment by absorption at 548 nm , the absorption maximum of the spiropyran moiety. An un-reacted mixture of protein and dye was also passed down a column to determine fraction separation elution profile.

4.3.3.c Protein Assay of dye protein conjugates

As the spiropyran dye adsorbs strongly under UV the concentration of spiropyran conjugated proteins cannot be determined by measuring the adsorption at 280nm. Determination of the conjugated protein content was achieved by using the modified Lowery assay (sigma code: P5656) method. The Lowery procedure has been found to be the most reliable and fairly straightforward assay for quantification of soluble proteins. In brief, a portion of the protein solution is made up to 1 ml with RO water. A 1 ml aliquot of RO water was used as a blank. 1ml of Lowery reagent solution is added to the standards and samples tube and is left to equilibrate at room temperature for twenty minutes. With immediate and constant mixing, 0.5 ml of Folin & Ciocalteu's Phenol Reagent was added to each solution and incubated at 20 °C for 30 minutes for a deeper colour to develop. At the end of incubation the samples were read against the blank at Abs_{740 nm}.

Measuring the absorption of a protein solution at 280nm is commonly performed as a non-destructive analysis method for protein concentrations. The aromatic rings present in the amino acid residues within the protein structure are responsible for the absorption at 280nm. As the spiropyran dye also absorbs strongly under UV illumination this method for protein determination was not suitable, as the dye absorption would mask the protein absorption affect.

4.3.3.d Photo-modulation of Horseradish peroxidase in solution

Horseradish peroxides was modified with spiropyran dye (*section 4.3.3a*) resulting with a protein: dye ratio of (9:1). The solution was kept in the dark for 24 hours to allow sufficient equilibrium time for dark adaptation. Taking a quartz cuvette, 1ml of solution (1mg/ml) was placed in the spectrometer with MES buffer (pH 6.8) as blank and 1 ml of ABTS was then placed in the cuvette and scanned immediately every 5 second for 3 minutes. This assay was performed again as above but with the same concentration of SP- HRP solution exposed to visible light (*Phillips tungsten filament bulb 80W*) or UV (*UVG-54, UV products Ltd, San Gabriel, CA. Peak output: 245 nm, 0.12 mps at 220 volts*), light source 10cm from each cuvette for 30 minutes prior to ABTS addition and scanning. The same procedure was carried out for native un-conjugated HRP for comparisons. All of the measurements were performed in triplicate. Native HRP (1mg/ml) and SP dye(1mg/ml) was dissolved together but not reacted and assayed as above this was also done in triplicate.

4.3.3.e Immobilisation of protein to modified micro titre plate

Pre-modified micro-titre plates were used to covalently attach bioactive proteins to the solid support to then be modified *in-situ* by covalent attaching the carboxylated spiropyran dye. To improve the binding capacity and reduce non-specific binding a carboxylated dextran matrix was attached to the surface of the aminated micro-titre plates. A 0.1% wt/vol solution of CMD (Fluka code 27560) made up in Analar water was activated by the addition of 2.5mg/ml EDC and 1mg/ml NHS. The activated solution was then pipetted in to each well (100µl) and left to incubate for 1 hour at room temperature. After incubation the plate was washed 3 times with Analar water.

A 200 µg/ml solution of protein of interest (HRP or monoclonal antibody) was made in 100mM MES buffer, pH 6.7 and 100µl of solution was pipetted into each well. These wells were then activated by the addition of 100µl of EDAC (2mg/ml)/NHS (2mg/ml) in pH6.7 MES buffer and the plate was shaken for 30 seconds and then was left to incubate at room temperature for 40 minutes. After incubation the plate was washed once in Analar and 0.05% Tween 20 solution and then three times in Analar water. Aliquots on 100µl of glycine (100mM) was pipetted into each well and incubated for a further 20 minutes to deactivate any un-reacted carboxyl groups of the dextran matrix left on the plate.

4.3.3.f Conjugation of spiropyran dye to the immobilised protein

A 1 mg/ml solution of the spiropyran dye was dissolved in MES buffer (25% ethanol in MES, 100mM, pH 6.7) and was activated by the addition of 1mg/ml EDAC and/or 1mg/ml NHS. 100µl of the activated spiropyran dye solution was pipetted into each well and allowed to incubate for 40 minutes at room temperature. At the end of the incubation the plate was washed four times in 100mM MES buffer.

4.3.3.g Immobilised Horseradish Peroxidase Photo-modulation Assays

Three strips of eight micro-titre wells coated with immobilised native HRP was placed in a micro titre plate holder with three strips of immobilised SP modified HRP wells (Immobilised protein attachment to micro titre wells was described in *section 4.3.3e and section 4.43f*). For blanks and control three strips of eight wells with only activated spiropyran dye (EDC/NHS) in the wells and three sets of strips with only MES buffer pH 6.8 was placed in the same plate holder. All the wells of the prepared plate was then flooded with PBS buffer and placed in a shallow water bath (1cm) and illuminated with *UV* or visible light. The lamps were held approximately 10 cm from the plate surface. The water bath was used as a thermal buffer to protect the enzyme against thermal heating from the light source. After illuminating each plate with either *UV* or visible light for ten minutes, the plate was immediately emptied of PBS and ABTS was added at 100µl per well and read at 405nm, every two minutes using a Dynex micro-titre plate reader (*Jencones Lab supplies, Bucks UK*). Control wells consisted of blank wells with no protein attachment and no dye and another with blank wells with dye but no protein.

4.3.3.h Immobilised Anti-body photo-modulation assay and ELISA assays

Three strips of eight micro titre wells immobilized with native anti-body and three strips of SP modified antibody wells were placed in a holder and flooded with PBS buffer (Immobilised protein attachment to micro titre wells was described in *section 4.3.3e and section 4.43f*). The micro titre plate, prepared as mentioned above and was placed in a 1cm water bath. The plate was then illuminated under *UV* or visible light for 10 minutes (the light source is placed 10 cm above the micro titre plate). After illumination under an appropriate light the PBS buffer was removed from the plates and 100µl of antigen-HRP at 100µg/ml was pipetted into each well. The plates are allowed to incubate for 25 minutes and then decanted immediately. Each plate was washed in Analar water (0.1% Tween 20). ABTS was then added to each well at 100µl per well and then read after 1-hour incubation at 405nm. Control wells consisted of blank wells with no protein attachment and no dye and another with blank wells with dye but no protein.

4.3.4 Photo modulation of antibody observed by SPR

50 μ g/ml of FITC-BSA was physically absorbed on the sensor surface. A solution of 1 μ g/ml of modified and native antibody was illuminated with UV or visible light held approximately 5cm above the cuvette filled sample solution. After 10 minutes exposure to the appropriate light source, 100 μ l of antibody was past over the surface of the sensor. The level of binding was observed and on rate recorded over 10 minutes.

4.4 Results and Discussion

4.4.1 Synthesis of the spiropyran dye

4.4.1.a The synthesis of the carboxylated spiropyran

To determine the successful synthesis of the carboxylated spiropyran (SP-COOH), the quaternary ammonium salt: 1-carboxymethyl-2,3,3-trimethylindolenium iodide, the precursor for the synthesis of SP-COOH and reaction compounds, was synthesised as given in section 5.6.2. The reaction compounds and dye product was dissolved in ethanol at 1mg/ml and dotted on a Silica oxide TLC plate and the plates were run in 100% ethanol.

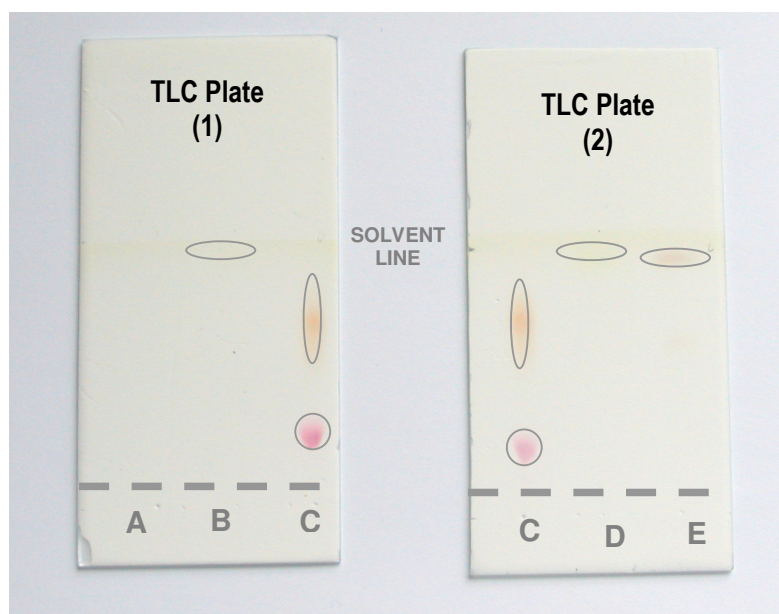


Figure 4.5: Photographic picture of the silica oxide TLC plate spotted with each component reactants for synthesising the carboxylated spiropyran compound. Where (A): 3-iodopropionic acid, (B) ,3,3-trimethylindolenium iodide, (C) 1-carboxymethyl-2,3,3-trimethylindolenium iodide, (D) 5-nitrosalicylaldehyde and (E) the spiropyran dye end product: 1-(β -carboxyethyl)-3,3-dimethylnitrospiro[indoline-2,2'-2,2'-H-benzopyraan.

Figure 4.5 and Table 4.1 shows that the 1-carboxymethyl-2,3,3-trimethylindolenium iodide had considerably different (R_f 0.304 and 0.703) profile values than the ether 3-iodopropionic acid (R_f of 1) which migrated along with the solvent line and 2,3,3 Trimethylindolinine (R_f 0.94). The ammonium salt also showed a more pronounced purple colouration that

became highly coloured when illuminated with UV light. The band at R_f 0.304 is believed to be 1-carboxymethyl-2,3,3-trimethylindolenium, with the band at R_f 0.703 being impurities in the product. The spiropyran dye product produce a clear single band at R_f 0.92, which was highly coloured when, illuminated in UV and faded quickly when exposed to visible light.

Table 4.1: Mean R_f value measurements of the reactant components for the synthesis of the carboxylated spiropyran dye

Plate Key	Compound name	Mean R_f Value
A	Iodopropionic Acid	0.81
B	2,3,4 Trimethylindoline	0.94
C	Ammonium Salt	0.304; 0.703
D	5 Nitrobenzaldehyde	0.94
E	Spiropyran dye (SP)	0.92

The different colouration (i.e. deep purple) under illumination with UV light of the spiropyran dye to its precursor compound (dark pink) and different R_f profile values indicated the purity and effective synthesis of the dye was achieved. This initial chromatography analysis illustrates the near purity of the end product and proof of effective synthesis of the carboxylated spiropyran dye. From the TLC analysis it would show that the synthesized product was sufficiently pure.

4.4.1.b Mass spectrometric analysis and Melting Point analysis of synthesised spiropyran product

The melting point of the synthesised spiropyran dye was taken and found to be 199.2-199.3°C; this was in accord with that given in the literature (*Arai et al., 1996; Kirkham, 1996*). The confirmation of the synthesized product was confirmed by mass spectra (*see figure 4.6*).

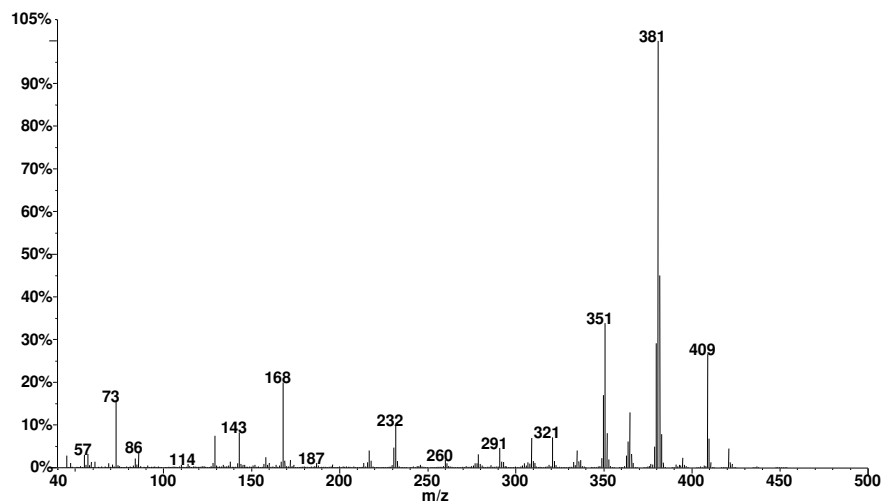


Figure 4.6: Mass Spectra of Spiropyran dye: Instrument Resolution: 6,000, Theoretical Mass ($C_{21}H_{20}N_2O_5$): 381.14504 ($M+H$), measured Mass: 381.14472. Error: 0.84ppm. Results interpreted by John Hill, Kent Mass Spectrometry.

4.4.1.c Summary of dye synthesis section

The synthesis of the spiropyran dye from the data given by TLC analysis, Mass spectroscopy and the dyes melting point all indicate that the desired product was achieved and the purity was high. The yield of the precursor and the final end product was 80% and 90% respectively of the starting weight. The TLC analysis with silica oxide plates showed good separation with ethanol (100%). Analysis with the aluminium fluorescence plate allowed the plate to be inspected under UV to determine the position of the uncoloured compounds and photo switching confirmation of the dye. Due to limited time and laboratory restrictions an NMR analysis was unable to be performed. NMR analysis would have given added confirmation on the compound functional groups.

4.4.2 Spiropyran dye characterisation

4.4.2.a Photo chromic activity of SP-COOH in solvents of different polarity

The syntheses spiropyran dye was assayed in different solvents. Solutions of SPCOOH (100 μ g/ml) were made up in ethanol, methanol and MES buffer.

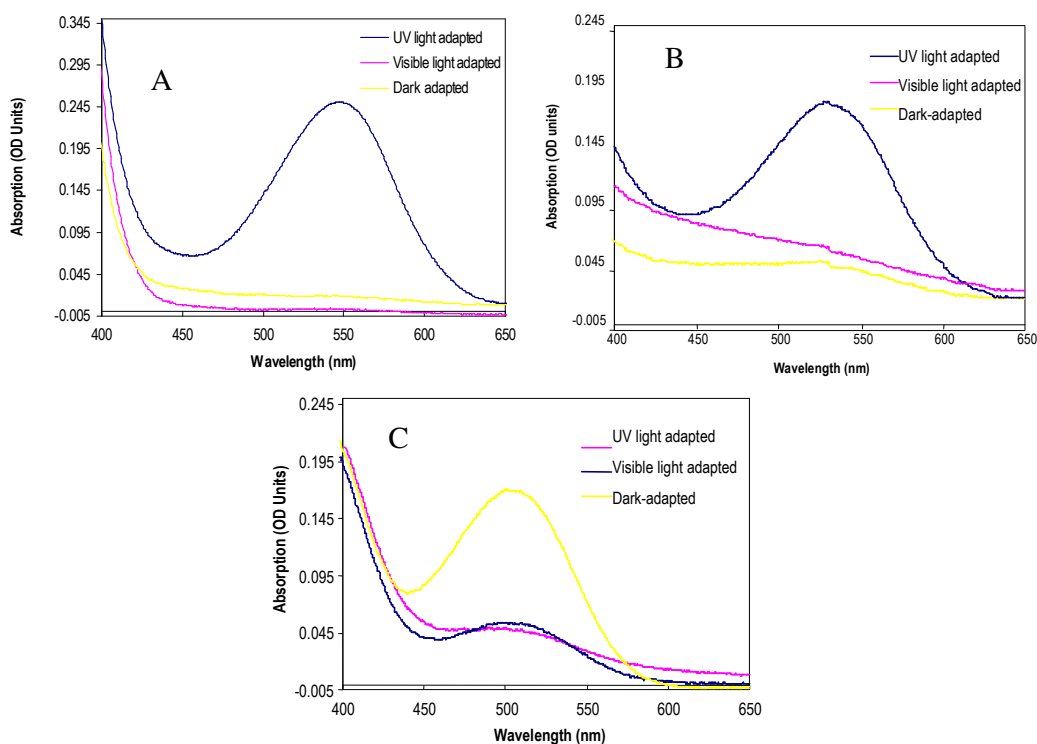


Figure 4.7 Absorption spectra of SP-COOH under Visible, UV illumination and dark adaptation. A) Ethanol (100%), B) methanol (100%) and C) MES buffer (pH7.1). The sample solution containing 100 μ g/ml SP-COOH was assayed after an initial 24 hours of dark adaptation and then illuminated with UV light or visible light for five minutes before being scanned (400-650nm).

Figure 4.7a shows the absorption of the SP-COOH in ethanol under different lighting regimes to have very different absorption profiles. The dark adapted solution showed a higher baseline than the visible baseline and therefore suggests that under visible illumination of the solution after UV radiation was able to drive the equilibrium of the merocyanine form further down towards the spiropyran form than its initial baseline. Illumination of UV showed intense coloration and absorption at 550nm of the solution indicating the change of the colourless spiropyran form to the merocyanine isomer form.

Figure 4.8b shows the absorption profile of the spiropyran in methanol. The peak absorption for the merocyanine isomer form in methanol has shift slightly to 530nm and the absorption peak is lower than for ethanol. The dark adaptation baseline is higher. By illuminated with visible light the merocyanine form is driven down however this is not as dramatic as the profile seen for ethanol. It would suggest that the equilibrium of the merocyanine and spiropyran forms were at a particular equilibrium in a racemic solution proportions existing in both forms.

Figure 4.7c shows the absorption spectra of SPCOOH in MES buffer. The profile show reverse photo-chromism compared to SPCOOH in methanol and ethanol. The dark adaptation of the solution absorption profile showed that the merocyanine form existed. The illumination of visible light drove the merocyanine form to it spiropyran form. The absorption peak in the dark form was at 510nm. Further illumination of the solution by UV irradiation showed a slight increase in absorption.

4.4.2.b Fade back Photo-chromic activity of the Spiropyran dye

Making a solution of the dye in 100% ethanol and scanning the sample between 400 and 650nm with an UV/visible spectrophotometer assayed the photochromic activity of the synthesised spiropyran. To determine the natural fade back of the merocyanine form to the spiropyran form, a 1 ml aliquot of 1 mg/ml SP-COOH in ethanol was illuminated for 15 minutes with UV light. The sample was then scanned every two minutes until the absorption profile stabilised. Natural fade back of the spiropyran form to the merocyanine form in MES buffer was also investigated using the same procedure but instead of illuminating with UV the solution was illuminated with visible light.

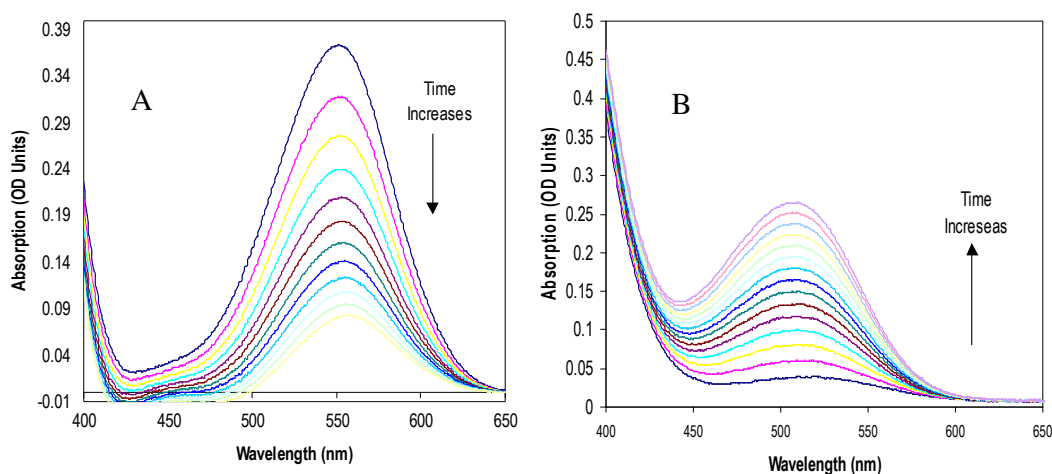


Figure 4.8(a+b): Fade back profile of the synthesised dye. A) Show the merocyanine form of SP-COOH converting to the spiropyran form. 1.0 ml of 1 mg/ml SP-COOH in ethanol was illuminated for 15 minutes with UV light and allowed to fade back to the spiropyran form. B) Shows reverse photo-chromism of the SP-COOH form converting to the merocyanine form. 1.0 ml of 1mg/ml of dye in MES The absorption profile was illuminated for 15 minutes with visible light and allowed to fade back to the merocyanine form recorded at two-minute intervals.

Figure 4.8 a+b shows normal and reverse photo-chromism fade back of the spiropyran form of SP-COOH to the merocyanine and visa versa. 1.0 ml of 1 mg/ml SP-COOH in ethanol and MES was illuminated for 15 minutes with UV of visible light and allowed to fade back. The absorption profile was recorded at two-minute intervals. Figure 4.8a show the natural fade back of the merocyanine form to the spiropyran form, it was observed that for the absorption profile to reach the initial baseline plateau level took 30 minutes in the dark. Figure 4.8b shows the reverse photo-chromism behaviour of the dye in MES buffer that had been illuminated with visible light and then left to adapt in the dark switching from the spiropyran form to the merocyanine form.

For the spiropyran dye to dark adapted in MES buffer after illumination with visible light took 30 minutes to reach 68% of the adsorption peak showed by the dye in ethanol. This would suggest that the natural fade back is a slightly slower process and extra energy provide by illumination from UV light is able to increase the intensity of adsorption. It was also noted that the absorption peak in the different buffer systems changed. In with ethanol the absorption peak was found to be at 550nm and in MES buffer at 510 nm. The information provided from examination of the dye in different buffers was used to optimise conditions for further work on protein-dye conjugate systems.

4.4.2.c Fatigue assessment

To assess whether the dye showed any fatigue when constantly being changed from one isomer to another the spiropyran dye was put through five cycles of UV and Visible illumination. Results obtained showed that for five illumination cycles the dye displayed very little to no degree of fatigue.

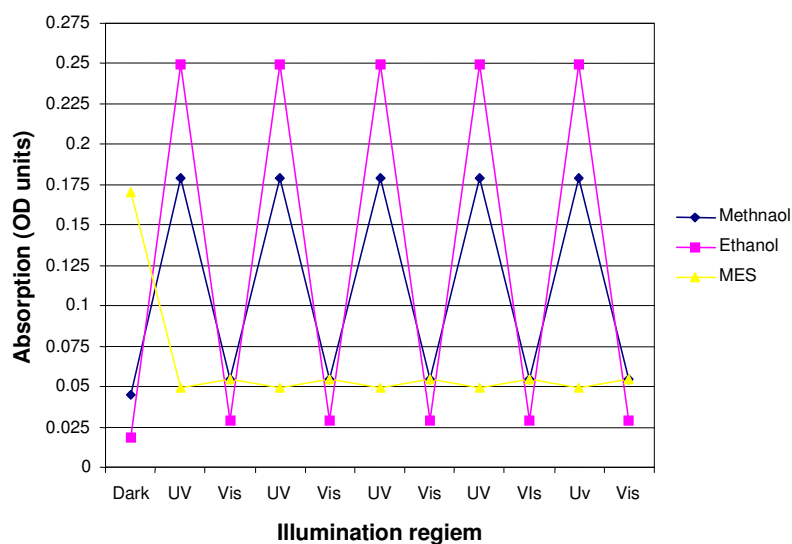


Figure 4.9: Fatigue assessment of spiropyran dye switching under different illuminations. 1mg/ml of spiropyran dye in solution of appropriate buffer (ethanol, methanol and MES) was repeatedly illuminated with alternating wavelengths of UV and Visible light and scanned consecutively for five cycles.

Absorptions for the dye were read at 550nm for ethanol, 530nm for Methanol and 510nm for MES.

4.4.2.d Summary of initial dye performance

From the results obtained in this section it was observed that the Spiropyran dye shows very different characteristic in different solvents. In ethanol the spiropyran displays normal photochromism where the dye is colourless in visible light and becomes highly coloured when exposed of UV light. In methanol, which is a slightly more polarised solvent, there is a shift in the adsorption peak from $550nm$ to $530nm$. The spiropyran dye showed complete reversal of the photo-chromic dye in MES buffer this is due to the polarised nature of the aqueous solution being able to stabilise the merocyanine form. The dye was investigated for fatigue and from the results obtained no fatigue could be seen. Total switching of the dye from one isomer to another could be achieved by exposure to an appropriate light source for 10-15 minutes.

4.4.3 Photo modulation of bioactive proteins

To determine whether spiropyran (SP) dyes can be used for the photo modulation of bioactive proteins, a simple, non-aggressive procedure of conjugating the dye to a protein to avoid any disruption to the protein structure and keep protein activity integrity, water-soluble EDAC reactions have been used to covalently attach spiropyran dye to proteins via the carboxyl group of the carboxylated spiropyran (SP-COOH) and the amino groups present on lysine residues and N-terminal amines of the protein primary structure. The spiropyran dye was covalently attached to horseradish peroxidase to determine whether covalent attachment was possible and to determine whether the conjugated spiropyran could affect the affinity of biological recognition molecules for their substrate, a comparison of the conjugated spiropyran protein specific activity and that of the native HRP under the influence of UV and visible light was conducted.

4.4.3.a Synthesis of the Spiropyran dye to Horseradish Peroxidase (SP-HRP)

SP-HRP was synthesised as given in *section 4.3.3a* and the elution profile of the conjugate is shown in *figure 4.10*, from this result, fractions 4, 5 and 6 are pooled and were used for subsequent experiments.

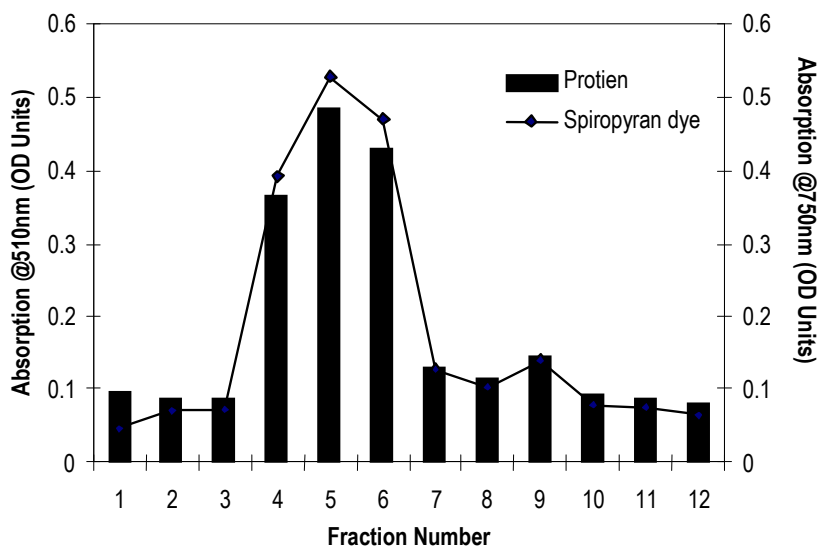


Figure 4.10: *Elution profile of SP-HRP fractions from a PD10 column and protein content-fraction volumes 1ml. The absorption of the spiropyran dye was measured at 510nm and protein content at 750nm (Lowery and Folin). The first three fractions are the initial void volume fraction that is usually discarded.*

The SP-HRP absorption profile shows the conjugated SP-HRP being eluted immediate after the void volume of the PD10 column. It was observed that there was little evidence of a second peak for un-conjugated spiropyran at fraction (9). Therefore, all the available spiropyran dye was conjugated to the protein or remained retained on the column. *Figure 4.11* shows the elution profile of the spiropyran dye at the same concentration passed down a PD10 column.

It was noted that as the protein separation was done in ambient light it was possible that any dye left on the column could have been bleached by the visible ambient light.

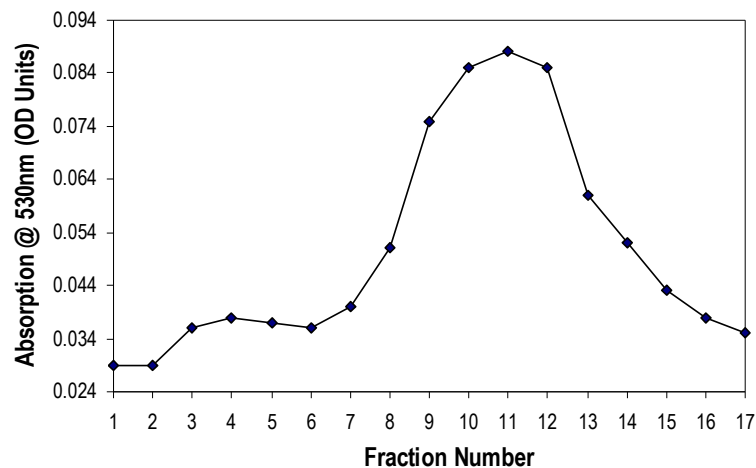


Figure: 4.11: Elution Profile of SPCOOH from a PD10 column- fraction volume 1ml. The absorption of the spiropyran dye was measured at 510 nm. The desalting PD10 column retained the low molecular weight dye well past the 6th fraction, which is usually, were higher molecular weight proteins are eluted first.

The elution profile of the dye shows that the dye was retained on the column and eluted after the ninth fraction with a peak occurring at the eleventh fraction. By comparing the results obtained from *figure 4.10* it can be concluded that most of the spiropyran dye has been conjugated to the HRP as the elution profile for just the protein is similar to the conjugated SP-HRP. Fractions Four, five and six of the covalently attached SP-HRP conjugate were pooled and the solution were assayed for protein concentration and spiropyran concentration.

4.4.3.b Photo-modulation of native HRP and SP-HRP in solution after exposure to Visible and UV illumination

To determine the photo modulation of the spiropyran conjugated HRP (SP-HRP), unmodified HRP was used for a control. The concentration of native (i.e. non modified protein) and modified sp-HRP was at 1 μ g/ml.

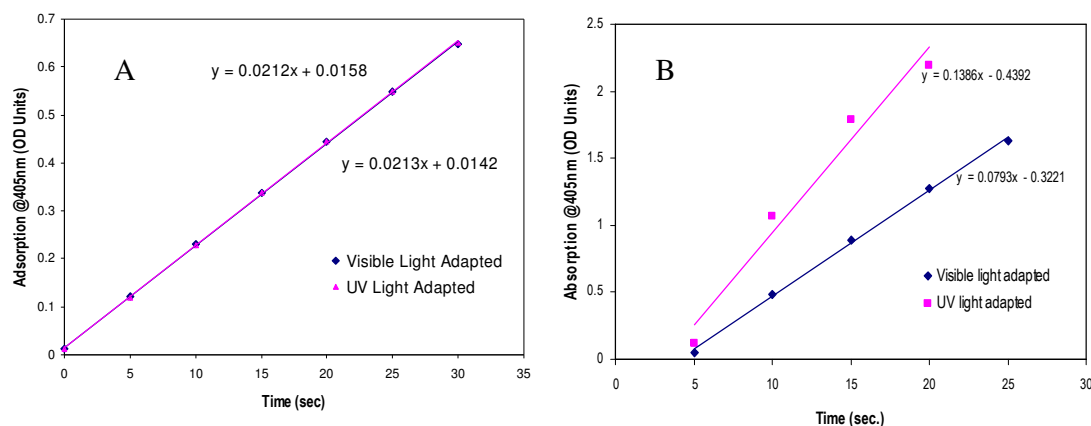


Figure 4.12 a+b: The Effect of UV and Visible illumination on native [A] and modified SP-HRP [B]. HRP at concentration of 1 μ g/ml in 100 mM MES, buffer 6.8 pH and ABTS substrate. The data points are absorbance measurements at 405 nm and the line represents linear regressions used to calculate the specific enzyme activity of the native and modified HRP under the different illuminations. Each data point represents the mean of three scans.

The native HRP (please refer to figure 4.12a) shows almost identical specific activity rates when illuminated with either UV or visible light (0.0212 and 0.0213 respectively). Therefore, no photo modulation of the natural native enzyme occurs when exposed to visible or UV light i.e. no discernable difference between the oxidation rates of ABTS substrate. The results show that for the modified HRP-SPCOOH the enzyme specific activity increased by 17% of that of the native HRP under visible illumination. The data for the modified SP-HRP showed that under illumination with UV light there was an increase of in enzyme activity by 79 %. This large variation in activity between the two illuminations compared to the native HRP indicates a large photo-modulation of the modified HRP occurring solely due to the introduction of the spiropyran dye.

4.4.3.c Photo-modulation of immobilised native and SP-HRP on micro-titre plates after exposure to visible and UV illumination

To ascertain whether biologically active proteins could be immobilised on solid phase supports and photo-modulated. HRP was used again as a model protein. The activity of the immobilised protein was estimated again using ABTS substrate.

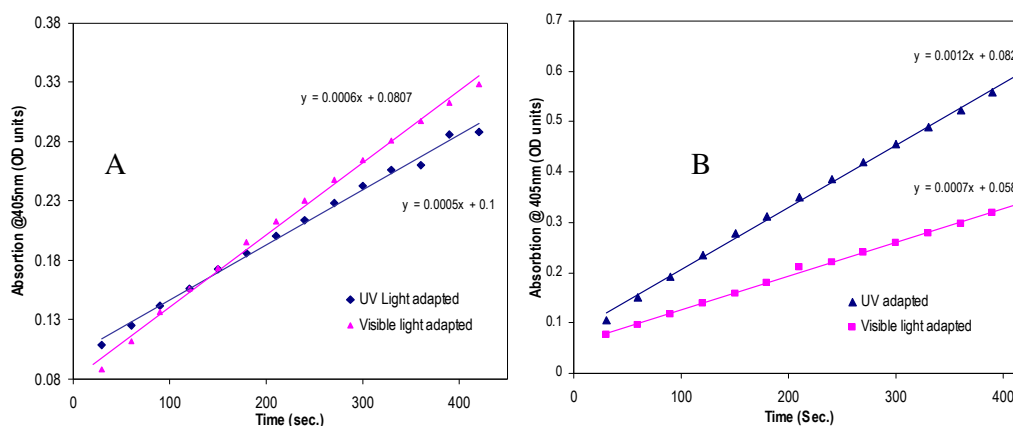


Figure 4.13(a+b): Immobilised native HRP and modified SP-HR photo-modulation of under UV and Visible illumination. Visible illumination (100watt) tungsten light at 5cm from surface, using a 1 cm deep water bath as a thermal buffer. The plate was read every 2 minutes. Each data point represent mean of 24 repeats with blanks subtracted.

The results show that the enzyme activity for the immobilised Native HRP is still conserved. Under exposure to visible and UV light a slight decreases in activity can be seen under UV light which is most probably due to the protein being adversely affected by the UV exposure. The difference in the two slopes shows a 20% drop from visible to UV.

The immobilised modified HRP has retained its activity and under visible illumination a 20% increase of activity can be seen compared to native immobilised HRP. There is approximately a 72% increase in enzyme activity for the modified SP-HRP when illuminated with UV light compared to the plate exposed to visible light.

4.4.3.d Summary of Photo-modulation of HRP

Photo modulation of bioactive protein of horseradish peroxidase by the attachment of spiropyran dye to its structure was proved to be possible when the SP-HRP was free in solution and also when covalently immobilised on micro-titre plates. The enzyme-dye conjugate upon exposure to *UV* irradiation showed a marked increase in activity of SP-HRP in solution and immobilised showing 79% and 72% increase respectively. To determine whether the presences of the dye was not acting as a substrate or enhancer, un-reacted native HRP mixed with SPCOOH dye and hydroperoxide was used as a control this confirmed that the dyes presence was not acting as an enhancer nor a substrate and that any increase in the enzyme activity was due to covalent attachment of the photosensitive dye modulating the protein activity.

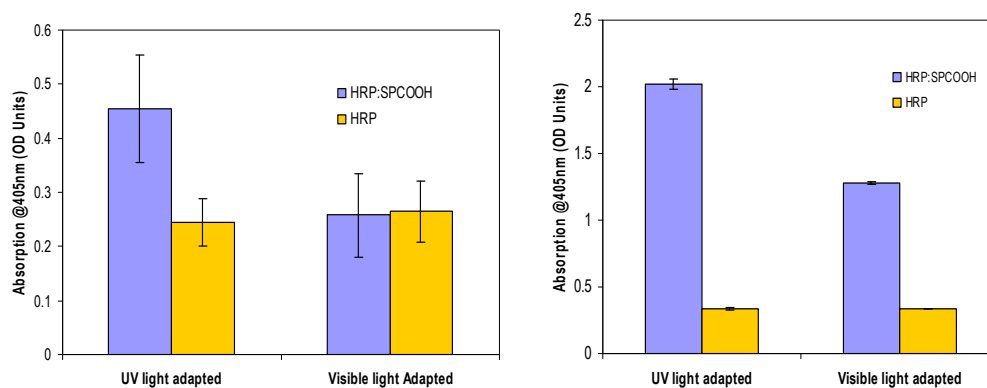


Figure 4.14 (a + b): Bar chart showing the activity of immobilised native and modified SP-HRP under different wavelengths illumination. A) HRP and SP-HRP in solution. B) HRP and SP-HRP covalently attached onto micro titre plate. Error bars represent the 1 standard deviation of 3 repeats for measurements taken in solution and 24 repeats on micro-titre plates.

The summary bar charts in *figure 4.14* clearly show that there is a marked difference between the specific activities of the modified HRP compared to native HRP under *UV* illumination.

4.4.4 Photo -modulation of antibodies

4.4.4.a Covalent attachment of spiropyran dye to antibodies

The procedure to covalently attach carboxylated spiropyran dye to an enzyme was very successful using the water based EDC reaction. Conjugation of the dye was then attempted on antibodies. Spiropyran dye was covalently attached to monoclonal anti-rabbit IgG and anti-FITC IgG antibodies.

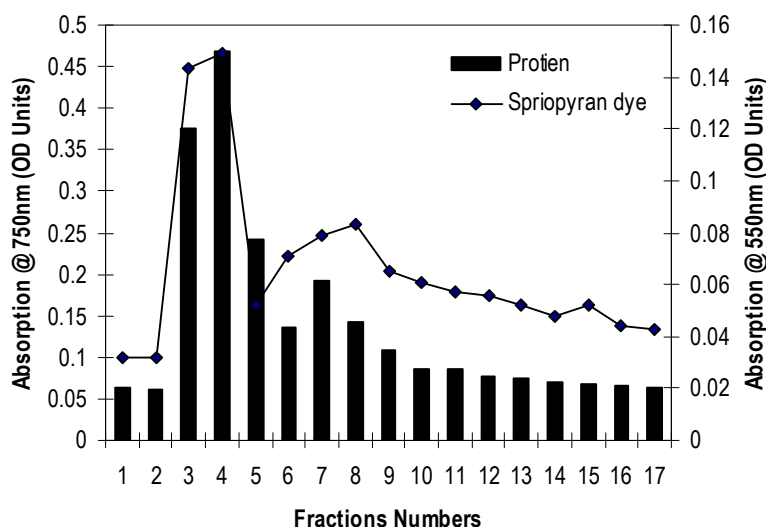


Figure 4.15 Elution profiles of SP-anti-FITC fractions from a PD10 column and protein content- fraction volumes 15.ml. The absorption of the spiropyran dye measured at 510nm and protein content at 750nm (Lowery and Foilin). The first two fractions are the initial void volume fraction that is usually discarded

The results show that the conjugation of the Spiropyran dye to the antibody was conjugated successfully. Elution of the antibodies followed immediately after the void volume of the column and fraction 3 and 5 were pooled together and used for further analysis. These fractions were chosen as the elution profile of the spiropyran dye also indicated that it had a high conjugation attachment. As would be expected the protein peak decreased with further elution and a second peak of spiropyran dye is seen at fraction seven and eight. Indicating elution free unattached spiropyran dye.

Conjugation of anti rabbit IgG antibody was not successful as the elution profile was very erratic. It was observed that sedimentation of the protein occurred. This suggested that by covalently attaching the dye to the protein made the antibody more hydrophobic and or destroyed the integrity of the protein structure to such a degree that aggregation and sedimentation occurred and was precipitated out of solution. Re-suspending the protein solution in 25% ethanol and MES buffer did not improve the solubility. The antibody was not used for further investigation. Due to small quantities of available monoclonal 17β Estradiol antibody, conjugation of free antibody in solution was not performed.

4.4.4.b Characterisation of modified anti-FITC antibody covalently attached to the spiropyran dye.

Spiropyran dye modified monoclonal anti-FITC (SP- α FITC) antibody in MES buffer was allowed to dark-adapt overnight after conjugation at 4°C. The antibody was then taken and 1ml (100 μ g/ml) was placed in a cuvette and scanned between 400-600nm. The antibody solution was then exposed to either UV of visible light for ten minutes and then scanned immediately after exposure.

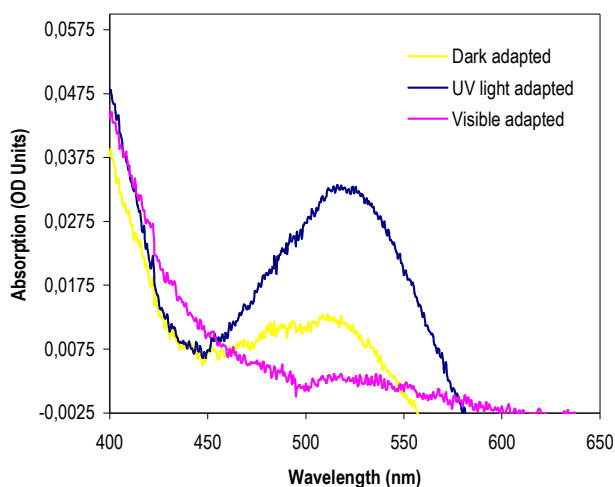


Figure 4.16 Absorption spectra of SP- α FITC in MES buffer under visible, UV illumination and dark adaptation. The sample containing anti FITC (100 μ g/ml) was assayed after an initial 24 hours of dark adaptation and the illuminated with UV light or visible light for five minutes before being assayed for adsorption

The absorption spectra of the α -FITC modified antibody (*please refer to figure 4.16*) showed that in MES buffer the merocyanine isomer exists when the protein solution was exposed to UV illumination and when dark-adapted. The spiropyran form only exists when exposed to visible light (i.e. absence of adsorption peak at 530nm). This shows a different absorption profile to the dye on its own in ethanol or MES buffer (*please refer back to figure 4.7*). This feature would suggest that the merocyanine form exists when attached to the protein as the antibody is ionised enough to stabilise the merocyanine moiety. This phenomena although not well described was reported by (*Harada et al., 1994*).

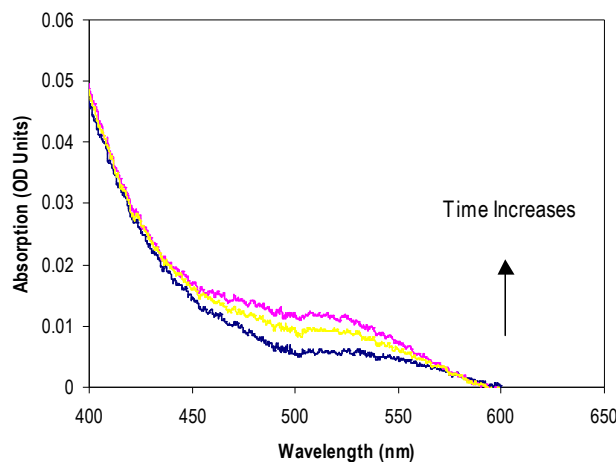


Figure 4.17 Fade back of the spiropyran form of SP- α FITC to the merocyanine form. 1ml of 100 μ g/ml SP- α FITC in MES was illuminated for 15 minutes with visible light and allowed to fade back to the merocyanine form. The absorption profile was recorded at two minute intervals. The scan represents 2, 15 and 30 minutes.

The natural fade back of the SP- α FITC took thirty minutes to reach approximately 30% of the absorption of the dark adaptation baseline. It was estimated that it would take the protein solution over two hours to revert back down to the dark adaptation baseline equilibrium.

4.4.4.c Summary of conjugation spiropyran dye to antibodies

The conjugation of the spiropyran dye to monoclonal anti-FITC was performed successfully. Photo-modulation of the modified antibody showed reverse photo-chromism and the presence of the merocyanine form existing under UV

exposure and dark adaptation. The adsorption peak for the dye conjugated protein was found to be at 510nm.

4.4.5 Development of the micro-titre plate-based immunoassay

It has been demonstrated that HRP could be successfully immobilised on to aminated micro-titre plates and conjugated *in situ* with the dye. For the development of a micro-titre immunoassay, antibodies were immobilised on to micro titre plates and then modified with the attachment of spiropyran dye. This was done to ascertain whether photo-modulation could be achieved and applied to an immunoassay format. The end objective was to assess whether photo-modulation was possible for biosensor surface regeneration.

4.4.5.a Photo-modulation of micro titre plate immobilised native and Spiropyran dye modified antibodies

Initially the antibody was covalently attached directly on to the modified aminated micro-titre plate using EDC at 30mg/ml. The use of EDC was used so that covalent attachment could occur under mild conditions to avoid extensive cross-reaction of the protein. The immobilised antibody was then covalently modified with the spiropyran dye (anti-FITC dye modified antibody [SP- α FITC] and anti- 17β Estradiol antibody [SP- α 17 β E₂]).

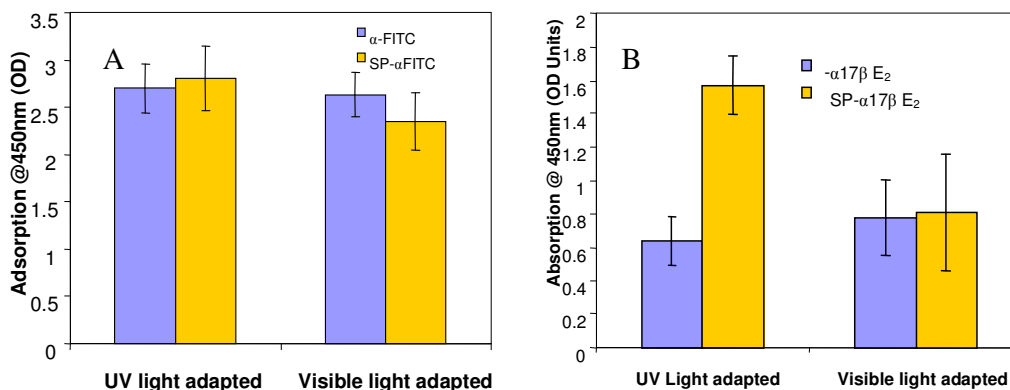


Figure 4.18 (a+b): The effect of photo-modulation on native and SP modified antibody under UV and visible illumination. A) Anti-FITC, B) anti-17 β estradiol. ABST assay was allowed to incubate for 15 minutes at 20°C before being stopped by the addition 100 μ l of 2M HCl.

Figure 4.18a shows the variation in binding affinity for native anti-FITC antibody to the FITC-HRP is very small and that the binding in terms of the absorption intensity is not discernibly affected by the exposure with different wavelength illumination. Conversely, there is a marked difference of antibody affinity to the FITC-HRP with the modified SP- α FITC antibody between the two different illuminations. The variation of antigen binding has reduced by 20% from the UV exposed plate to the visible. This would suggest that the dye when switched to the spiropyran form by visible light has less affinity to the labelled antigen than when it is in the merocyanine form *i.e.* under UV illumination the antibody is in its “on” state and under visible light the antibody is in its “off” state.

For the monoclonal anti- 17β Estradiol (*please refer to Figure 4.18b*) the result show that under visible light there is very little absorption variation in affinity binding between the two different illumination for the native anti- 17β Estradiol and that of the modified anti- 17β Estradiol under UV light. However, a large increase in binding has occurred in the modified antibody under visible illumination. The increase in illumination is $\sim 100\%$ more than the UV illuminated modified anti 17β Estradiol IgG. For this antibody system the results would suggest that the attached dye increases the affinity of the antibody to its antigen when the dye is in its spiropyran form. This maybe due to the binding site being more attractive to the highly hydrophobic 17β Estradiol molecule as the dye is increasing the hydrophobicity of the antibody binding site. Therefore, the 17β Estradiol antibody is in its “on” state when illuminated by visible light and in its “off” state when illuminated by UV or dark-adapted.

4.4.5.b Variation of conjugation reaction time and conjugation reagents

To determine the optimum reaction time of the conjugation of the spiropyran dye to the immobilised antibody in terms of dye loading and subsequent photo modulation the contact time of the dye was varied at regular intervals. The conjugation reagents were also varied to determine whether it would affect the extent of SPCOOH loading with time.

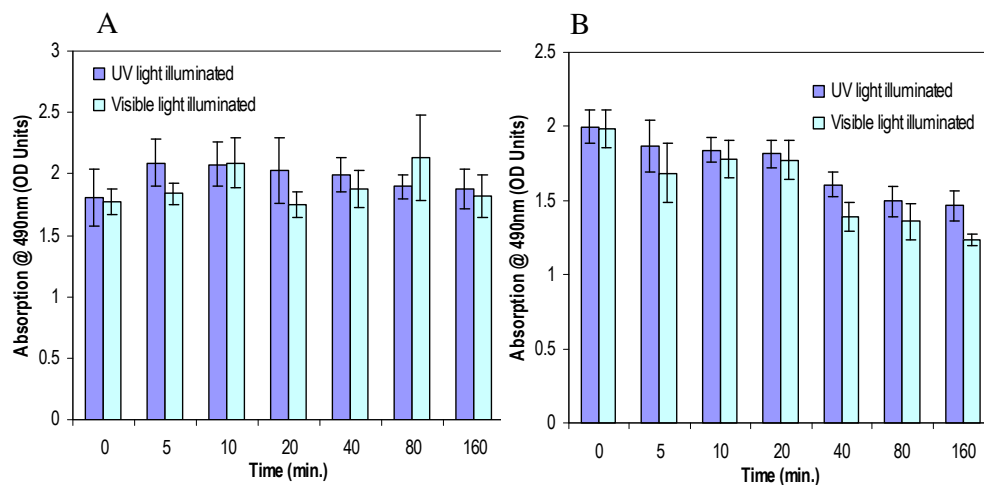


Figure 4.19 (a+b): Conjugation reaction time effect of on the photo-modulation of antibody affinity to FITC-HRP. Conjugation was performed using A) EDC and B) EDC/NHS to activate the Spiropyran dye (SP) before the solution was pipetted into the micro titre wells for covalent attachment to the immobilised antibody. Error bars represent the standard deviation of eight repeats with blanks subtracted.

Conjugation of the dye with EDC showed a highly erratic time profile and it would suggest that most of the dye attachment occurred in the first 10 minutes. From the data obtained it was deduced that the optimum reaction time and conjugation reagent used was using EDC/NHS and reacting between 30-40 minutes as it showed the most photo-modulation difference between each illumination with close standard deviation. However, the results obtained from EDC/NHS show a decrease in relative antigen affinity with time this clearly indicates that EDC/NHS is a more stable intermediate and continues to covalently attach dye to the protein reducing the antibody binding integrity.

4.4.5.c Established Photo-modulated micro titre plate immunoassay

Using the information gathered in section 4.4.5(a and b) an initial direct immunoassay of different concentrations of labelled antigen was applied to the native and modified covalently attached antibody and illuminated at different wavelengths.

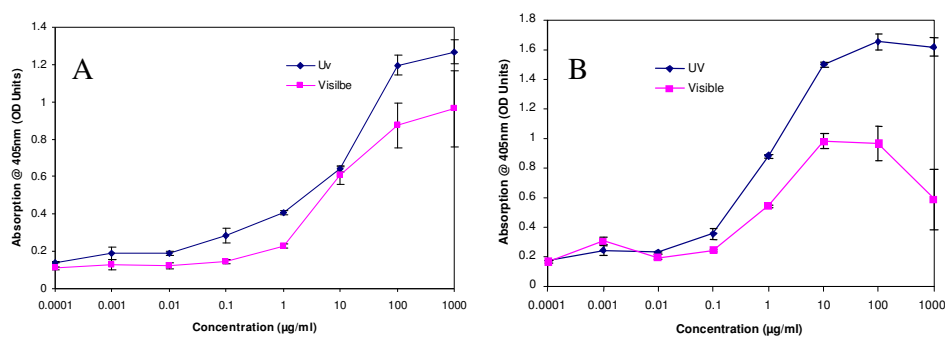


Figure 4.20 (a+b): Calibration curve for native [A] and modified SP- α FITC antibody [B] under UV and visible illumination. Anti FITC was covalent attached to a CMD modified aminated micro-titre plate. 100 μ l of FITC-HRP at varying concentrations was pipetted into individual wells. The plated was assayed with ABTS and stopped with 2M HCL after 20 minutes. Error bar represent 1 standard deviation of the mean of 3 repeats, with blanks subtracted.

The result in (figure 4.20 [A] and [B]) show very different calibration curve for native and modified SP- α FITC. It can be seen that for modified anti FITC the calibration profiled is very different from UV exposed plate and visible exposed plate. This agrees with the finding in section 4.4.5 were visible illumination decreased covalent attachment to the antigen. The binding has reduced between 55% and 70%.

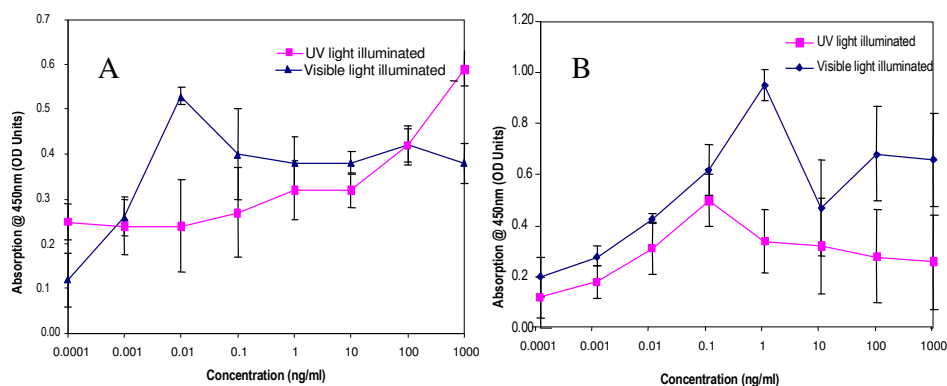


Figure 4.21: Calibration curve for native [A] and dye modified SP- anti Estradiol antibody [B] under UV and visible illumination. Anti-Estradiol antibody was covalent attached to a CMD modified aminated plate. 100ul of Estradiol -HRP at 100µg/ml was pipetted in to individual wells. The plated was assayed with ABTS and stopped with 2M HCL after 20 minutes. Error bar represent 1 standard deviation of the mean of 3 repeats with blanks subtracted.

The results for anti-Estradiol antibodies were not as conclusive as anti-FITC antibody and the calibration curve was very erratic. However, from the results obtained the calibration curve for native anti 17β Estradiol showed a saturation of the antibody at the surface at 0.01µg/ml. This may be due to very little antibody bound to the surface or denaturing of the antibody affected by the different illuminations. The result obtained from the modified anti-β Estradiol antibody shows a similar trend to that of previous data presented in section 4.4.5a and shows that there is an increase in antibody activity when exposed to visible light. There is also a marked difference in calibration curve between UV and Visible illumination, were at 1µg/ml there was a 200% difference in affinity binding.

4.4.5.d Summary of the development of a micro-titre plate-based immunoassay

In summary the result obtained in this study demonstrated that photo-modulation of covalently attached photo-chromic dye to antibodies could be achieved and applied to an immunoassay plate analysis. The different antibodies used in this study showed different photo-modulation characteristics. For modified anti-FITC antibody the photo-modulation displayed its “on” state when UV exposed or dark-adapted. For anti-17 β -Estradiol the photo-modulation “on” state was when it was exposed to visible light.

4.4.6 SPR time course data

The ultimate goal into investigating photo-modulation of bioactive proteins was to develop a photo-modulated immunoassay for the sole purpose of it being transfer onto the SPR analyser platform.

4.4.6.a Photo-modulation of spiropyran dye modified antibody probed by SPR analysis

To determine the level of affinity binding between native anti FITC and modified anti FITC/SPCOOH, 1ml of modified anti FITC in phosphate buffer (pH7.4) at 10 μ g/ml was passed over a surface prepared with BSA-FITC. This was compared with the binding of the native anti- FITC.

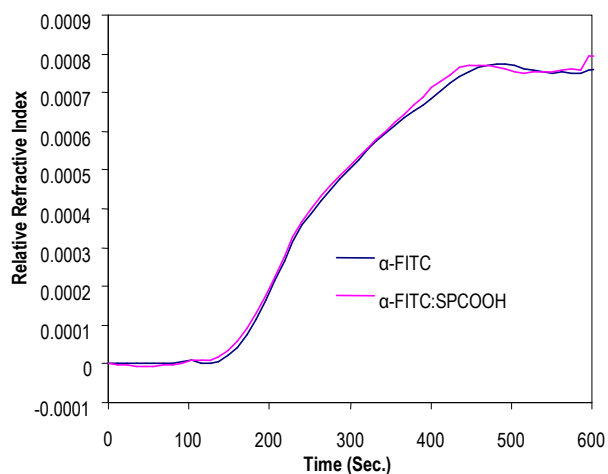


Figure 4.22: Affinity binding of anti-FITC and modified SP- Anti-FITC on a BSA-FITC prepared surface SPR time course data. 1ml (10 μ g/ml) was allowed to pass over the surface at 50ul/ml and then buffer was changed to PBS to wash any unbound protein off. Affinity binding was then calculated. The curves are composite curves of the mean of three samples for both antibody samples.

The results show that the affinity binding time course for a-FITC and modified anti-FITC/SPCOOH are very similar and very little variation in binding has occurred.

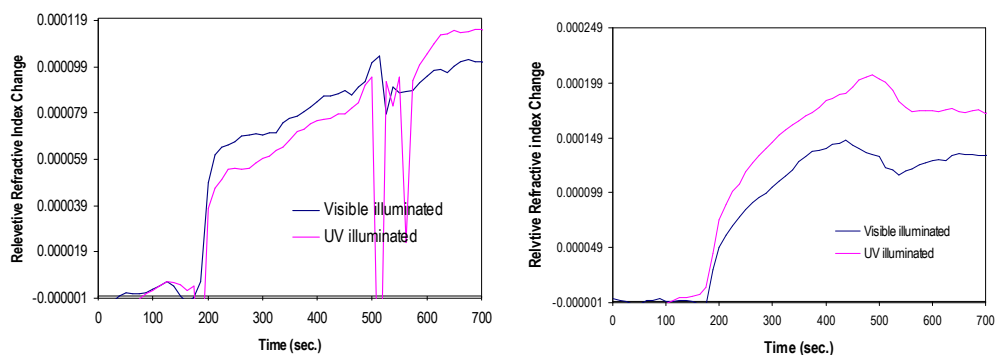


Figure 4.23(a+b): SPR time course sensorgram: showing affinity binding of native[A] and modified SP- α FITC [B] illuminated with UV or visible illumination before the binding event. 1 ml (100 μ g/ml) antibody solution was passed over a prepared bioactive surface of BSA FITC. At 500 second the antibody solution was exchanged with PBS to remove any unbound antibody and establish as new baseline.

The result shown in *figure 4.23a* shows the binding event of native anti FITC that has been illuminated with UV light and visible light for 20 minutes prior to passing over the bioactive surface of the SPR sensor. The binding curves are very irregular; this may be due the antibody being affected by the illumination. The time course for the modified SP- α FITC again displayed a marked difference in binding when illuminated by visible light, in accord with the findings in previous sections. There is a 33% decrease in binding under visible illumination compared to UV illumination.

4.5 Discussion and Conclusions

4.5.1 Synthesis and characterisation of the carboxylated spiropyran dye

The method used to synthesis the carboxylated SP-COOH was fairly simple to perform. By modifying the method used by Kirkham (*Kirkham, 1996*) a relatively pure substance could be synthesised with a high yield. To synthesis the dye in this project the washing step of the precursor was re-crystallised and filtered three times each time using a reduced amount of ethanol. This allowed the precursor ammonium salt to be dissolved in very small quantities of ethanol and the solid able to precipitate out at a relatively high purity once cooled. The slow re-crystallisation meant less washing and reduced contact and consumption of the solvents used to produce the precursor. The yield of the final product was approx 90% which is in good comparison with published methods (*Kirkham, 1996; Aizwa et al., 1977*).

TLC analysis of the reagent products and synthesised solid indicated at the purity of the synthesised product, further element analysis and melting point were all in agreement to the purity and complete synthesis of the desired end product. Due to limited time and laboratory constraints NMR analysis was unable to be performed. This would have confirmed the synthesised compounds structure and true identification.

The reverse photo-modulation of the spiropyran dye in aqueous media, was published by Namba et al (*Namba et al., 1975*) and it was felt important to investigate this further as most bioactive protein used in detection analysis are often carried out in aqueous environments. The spiropyran dye was completely soluble in ethanol and was then able to be made up in aqueous solutions very easily. It was observed that the visible absorption peak for free spiropyran dye in ethanol, methanol and MES buffer decreased with the polarity of the solvent. The absorption peak decreased and the photo-chromism reversed in 99.9% aqueous buffer. The isomerizable merocyanine form was stabilized in aqueous buffer as it is in a hydrolysed state.

4.5.2 Spiropyran dye conjugation to soluble protein

The aim of this study was to be able to photo-modulated Estradiol antibodies in order to apply them to the portable remote Spreeta™ analyser for the detection of endocrine disrupters in waste-water. Because of the limited supply of antibody it was first decided to demonstrate covalent attachment of the spiropyran dye to bioactive protein by using readily available horseradish peroxidase and anti-FITC. These two proteins have also been studied by Weston [1999], with the rationale to take his preliminary study a little further and be able to apply photo-modulation of antibodies into an immunoassay format for SPR sensing (Weston *et al.*, 1999b).

Horseradish peroxidase is a robust protein that is extensively used in immunoassays and biosensors development. It is useful as it produces a redox reaction to electrochemical sensors, as well as being used as a non-radioactive label for micro titre plate immunoassays. Weston (Weston, 1999) saw a decrease in activity by 53% due to covalent attachment of the dye to HRP, he suggested that due to dye attachment enzyme activity was inhibited. However, the results obtained in this study showed an increase in the HRP specific activity due to covalent attachment of the dye, both in solution and immobilized. To the extent of the publication research taken for this study it was found that this phenomenon had not been mentioned before. In a recent review publication on the stability of peroxidases, Azvedo *et al.* (Azvedo *et al.*, 2001) noted that higher activities of HRP can be obtained when immobilised to a solid support, than when free in solution. This suggests that covalent attachment of HRP onto a solid support was able to suppress to some extent the unfolding process of the protein, conserving the active site and stabilising the enzyme (Azvedo *et al.*, 2001; Kirkham, 1996). Therefore, the increase in enzyme activity seen in this study could be due to the dye having a similar effect. Where the conjugated spiropyran dye is influencing the enzyme's structure in such a way that it conserves the integrity of the enzyme structure and affecting the substrate turn over rate.

However, when the spiropyran dye modified HRP enzyme was exposed to UV the activity is seen to be even greater, this must be due to an opening effect caused by the presence of the highly conjugated merocyanine form. Being that the conjugated form may play a role in moving electrons in the enzyme substrate system and therefore increase the ability of the enzyme turnover activity. A brief study performed eight months after preparing a solution of spiropyran dye conjugated HRP, showed that the enzyme retained 90% of its activity compared to freshly prepared solution of native HRP. Conversely, a solution of eight months old native HRP showed no activity whatsoever (Data not presented). This further supports the hypothesis that the spiropyran dye

was able to stabilise the enzyme structure increasing the activity and stability. This finding would be of high value to affinity based systems where amplification of the detection signal is required and/or enzyme stabilisation is required. Horseradish peroxidase has nine amino groups in its structure that would be available for covalent bonding to the carboxylated spiropyran dye using EDC/NHS chemistry. It was estimated that all of the nine available sites were successfully bonded to a spiropyran dye molecule.

4.5.3 Photo-modulation and Immunoassay applications

Photo modulation of antibodies in solution and immobilised on solid surfaces was demonstrated to be possible. The initial study towards an immunoassay using photosensitive antibodies could be used to regenerate bio-sensing surfaces in a reagent-less way. Further investigation to this line of enquiry is required to confirm this position. However, previous studies on photo-reversible immunoassays have been demonstrated by attaching photosensitive molecules to the antigen (Kagner *et al.*, 1999; Blonder *et al.*, 1997; Kirkham, 1996; Harada *et al.*, 1994). This approach worked very well for their application. More recently two papers have been published detailing the incorporation of photoisomerisable non-natural amino acids onto proteins. The authors detailed the synthesis and characterisation of horseradish peroxidase mutants containing L-p-phenylazophenylalanine (azoAla) at different positions on the enzyme backbone. Synthesis was achieved by using an *Escherichia coli* in vitro translation (Simonian *et al.*, 2002; Muranaka *et al.*, 2002; Hohsaka *et al.*, 2002; Miyata *et al.*, 1999; Hohsaka *et al.*, 1994; Martinek *et al.*, 1978). Modifying antibodies and receptors properties via genetic engineering has been demonstrated to be a powerful tool (Hock *et al.*, 2002) and to be able to incorporate photo-sensitive moieties to the antibody structure may also be a great possibility. Further research in this direction may well realise the full and real potential of production and use of photo-controllable important proteins.

5 Final discussion, conclusions and future work

Chapter 5

Final discussion, conclusions and future work

5.1 Introduction

The research presented within this thesis has examined several areas towards the development of a portable sensing system that utilised the phenomena of SPR as a basis for the detection of endocrine disrupting chemicals present in waste-waters and surface waters. In addition, a special study has been made on the photo-modulation of bioactive proteins to create sensor interfaces which can be regenerated remotely, without the use of reagents and harsh chemicals. The fundamental points of this research are presented in this chapter are set out as a summary of individual chapters, followed by suggestions for further studies and future prospects.

5.2 Final discussion

5.2.1 Evaluation of the Spreeta™ sensor for the incorporation into a portable analyser

Over the course of the research presented in this thesis the Spreeta™ sensor was continuously being updated and improved. The sensitivity of the sensor has greatly improved and from the results obtained in this study it was concluded that the Spreeta™ sensor can be directly compared to the Biacore® 3000 system in terms of sensitivity. This finding is in agreement with a recent review where it was reported that Biacore® 3000 had a refractive index range of 1.33-1.40 and a limit of detection (resolvable refractive index change) of 3×10^{-7} and that of the Spreeta™ had a refractive index change of 1.33-1.48 with a limit of detection of 0.3×10^{-6} (Leonard *et al.*, 2003).

The integration of the sensor into a customised instrument is attractive and the result presented in this report demonstrates that it is highly suitable for field applications provided that all the necessary shielding and peripherals are incorporated. Commercially the sensor has been incorporated into a miniature hand held refractometer (*Apt Electronics, Litchfield, USA*) and is currently being developed into a bench top SPR instrument based on a 96 well assay format (*Prolinx Inc. Washington, USA*). These examples demonstrate the level of interest instrument developers have in the sensor. One of the main drawbacks of the sensor is that it has only one channel. Hence, assay development and sensor application was very difficult to perform as a lot of data compensation was required (i.e. subtracting non-specific binding time scan from measured data). Had there been a reference channel, non-specific binding and assay artefact probing could be determined easily and simultaneously. Naimushin *et al.* used a β prototype double channel Spreeta™ sensor for the detection of *Staphylococcus aureus* enterotoxin B in which they were able to detect concentration down to femtomolar levels (Naimushin *et al.*, 2002). This was possible due to the use of an amplification step and simultaneous reference channel reading and subtraction. Texas instrument are currently in the process of releasing a multi channel Spreeta™ sensor, which will be a 3 channel sensor said to be available in 2004. Nomadics Inc are also considering producing a bench top instrument that will have similar specifications as the Biacore® 3000. However, this will not be available until 2007 (John Wisehart, *personal communication*).

5.2.1.a Immobilisation of the biologically active sensing surface

Due to the unique design of the Spreeta™ sensor used in this study it was deemed important to find an appropriate way of immobilizing a bio-specific surface to the sensor and a way of regenerating the surface. The sensor had a recessed gold surface that made it very difficult to modify. Therefore, within this study the sensing surface was considered irreplaceable. As the sensor was also to be used as a field deployable analyser a simple surface modification step and regeneration protocol was developed. By physically adsorbing Ovalbumin conjugated to Estrone-3-Glucronide ligand at 50µg/ml onto the sensing surface, an *in situ* bio-specific monolayer could be prepared. This was found to be suitable for subsequent antibody binding assays. Other researcher using the Spreeta™ sensor also found that physical adsorption of there haptent-protein conjugate was appropriate for their assays (*Elkind et al., 1999; Kukanskis et al., 1999; Strong et al., 1999; Melendez et al., 1996*). Covalent attachment of target ligands to the surface was found to be very difficult in this study as the SPR dip resolution was unable to be fully resolved after surface modification. This was probably due to the immobilization method used and the added modified layer on the sensing surface having a high interfacial refractive index value that was near to or at the sensors refractive index limit (1.48). It has been reported that covalent attachment onto the sensor is possible (*Naimushin et al., 2002; Woodbury et al., 1998*). Therefore, a possible way of solving this problem would have been to try and reduce the amount attached to the surface and/or using a different liquid with a higher refractive index than water (e.g. 15% sucrose solution) for initialising the sensor. *Niamusin et al.,* and *Woodbury et al.,* were able to immobilize gold binding peptide.-alkaline phosphatases on to the gold surface as a foundation layer and then covalently attach recognition molecules (antibodies) to the protein (*Naimushin et al., 2002; Woodbury et al., 1998*).

5.2.1.b Sensor regeneration of the SPR sensor

Successful immobilization technology in biosensor design should yield a stable product with a high retention in affinity binding activity. Conventional homogenous and heterogeneous immunoassays, respectively work discontinuously. However, it is highly desirable that immunosensor devices, especially for environmental diagnostics, are capable of quasi-continuous recording. Immunoassay using high affinity antibodies allows for high sensitivity but not fast reversibility. High affinity and reversibility are mutually exclusive of each other (*Luppa et al., 2001*). The regeneration of the binding sites of the antibody bound to the immunosensor surface needs harsh procedures. The use of strong acidic or alkaline solutions or other known regeneration buffers are potentially harmful to the sensing surface and may

cause subtle baseline drift problems. There are many different approaches to solve the “anti-body regeneration problem e.g. displacement with high concentration of analyte or antibody (*Elkind et al., 1999*) or antibody /receptor attachment on the sensor surface with physically adsorbed protein A or protein G that captures the antibody in an orientated manner which in turn can be regenerated with a glycine /acid buffer (*Aoyagi et al., 2003; Suzuki et al., 2001*).

Surface regeneration of the sensing surface was tackled in this report by taking a novel approach and completely stripping the physically adsorbed biologically specific layer on the sensor surface, thereby enabling the sensor to be reused. The employment of Persil laundry detergent which contains a defined complex mixture of surfactants, proteases, cellulases, lipases and bleaching agents, proved to be an effective regeneration stripping buffer. It was also concluded that NaOH/Triton X100 regeneration buffer was effective at disrupting the anti-body / antigen binding releasing the immobilised antigen for subsequent assay binding. Several publications have used this method for regenerating their surface. This regeneration approach was found to be appropriate if wanting to use the sensor for only one type of antigen-protein conjugate immobilized on the surface (*Simonian et al., 2002; Elkind et al., 1999; Kukanskis et al., 1999; Strong et al., 1999; Melendez et al., 1996*). However, the complete stripping of the surface with Persil allowed for the surface to be available for different antigen- protein conjugate to be physically adsorbed on the surface making the sensor surface modification step easy and flexible. The physical adsorption of the biologically specific layer on the sensor surface also proved to be a very reproducible way of immobilising the antigen, where the protein layer formed a good surface coverage of the gold surface with little non-specific binding.

5.2.1.c Initial Indirect immunoassay for Oestone-3-Gulcuronide: a modal EDC

An indirect immunoassay was developed to detect Estrone-3-Gulcuronide (E_13G) as a model endocrine disrupter. This was achieved by physically absorbing E_13G -ovalbumin conjugate on to the sensing surface and then pre-incubating $0.25\mu\text{g/ml}$ of anti- E_13G IgG antibody with the sample. The pre-incubated sample was then allowed to flow passed the sensing surface. The amount of free antibody bound to the sensor surface (change in the refraction index baseline) is inversely proportional to the concentration of the analyte present in the sample. The assay calibration curve for the model EDC: E_13G showed good reproducibility and had a working range between $0.1\text{-}100\mu\text{g/L}$. This result compares favourably with other published SPR based detection of steroidal antigens which had working ranges of $0.5\text{-}50\mu\text{g/L}$ for progesterone

using Biacore® 2000 (*Gills et al., 2002*) and 0.976-62.50 µg/L for Morphine-3-Glucuronide (*Dillon et al., 2003*).

5.2.2 Development and optimisation of an Micro-titre plate ELISA and SPR analyser immunoassay for the detection of EDCs in environmental samples

The development of an indirect ELISA assay for the detection of 17 β Estradiol (E_2) was deemed to be important, as assay development and optimisation was required for the assay to be successfully transferred to both SPR platforms. Immunoassay micro-titre plate development allows for the optimisation of several parameters such as concentration of the coating protein buffer and antibody could be assayed and optimised at the same time rather than on the single channel sensor of the analyser. The developed ELISA assay, the Spreeta™ analyser immunoassay, the Biacore® 3000 immunoassay and a commercial Estradiol ELISA test kit were all used to obtain calibration curves in spiked buffered samples and spiked synthetic waste water samples. These curves were then used to calculate the concentration of spiked real waste water samples.

5.2.2.a ELISA Assay Development for 17 β Estradiol

The developed ELISA assay was designed to be directly transferable to the Spreeta™ analyser and Biacore® 3000 instruments. With this in mind the main requirements of the ELISA assay was the pre-incubation of the antibody and sample and physical adsorption of the analyte-protein conjugate to the micro-titre plate. It was also felt necessary to optimise the timescale that the ELISA was to be performed. It was observed that by leaving the pre-incubated sample and antibody within the wells for more than 20 minutes the sample equilibrium of the antibody preferred to be attached to the plate rather than to the free analyte. This may be due to the antibody preferring the antigen-protein conjugate binding site due to the surface creating a hydrophobic environment that the antibody prefers. The avidity of the anti-body may also be greater for the conjugate antigen than the antigen in the free solution. Sherry et al. (*Sherry et al., 1999*) noted a similar phenomena when they had vitellogenin coated on their plate and the polyclonal antibodies used in the assay preferred to bind to the plate then remain in solution. The working limits of the ELISA assay was 0.3-70µg/L

5.2.2.b Surface plasmon Resonance detection of 17 β Estradiol

The immunoassay performed on both SPR instruments showed good comparability with the Spreeta™ analyser having a calibration curve working range between 0.1-10 μ g/L and The Biacore® 3000 instrument having a working range of 0.1-10 μ g/L. Both of these working range are comparable to results published using SPR to measure estrogen hormones/mimics in water samples.(*Usami et al., 2002; Pearson et al., 2001; Nishikawa et al., 1999*). The performance of the SPR based immunoassay compared to the developed ELISA was very close to each other. It would have been expected that the ELISA assay with a labelled detection signal amplification would have been at least one magnitude more sensitive (*Luppa et al., 2001*). The reason why this was not seen is most probably due to the assay format. As the SPR based system required a constant but steady flow of sample over the sensing surface. Therefore, the re-establishment of the antibody equilibrium in contact with the surface as mentioned above (*section 5.2.2.a*) was not able to play a role. Therefore, greater surface sensitivity displayed in the SPR immunoassay format then the ELSIA based assay.

5.2.2.c Detection of Spiked samples in buffer, synthetic waste water and real waste water.

For all the samples measured on all of the immunoassay formats the recovery rates of the samples were within the acceptable range with the exception of one. The Association of Official Analytical chemistry states that recovery rate should be between 70-120%. It could therefore be concluded that the assay are able to be used sufficiently with good accuracy.

5.2.3 Remote sensing and regeneration of sensing surfaces using Photo-chromic dyes

5.2.3.a Spiropyran dye identification and characterisation

The method used to synthesis the carboxylated SP-COOH was fairly simple to perform. A modified method used by Kirkham (*Kirkham, 1996*) resulted in producing a relatively pure substance with a high yield. To synthesis the dye in this project the washing step of the precursor was re-crystallised and filtered three times each time using a reduced amount of ethanol. This allowed the precursor ammonium salt to be dissolved in very small quantities of ethanol and the solid able being to precipitate out at a relatively high purity once cooled. The yield of the final product was approx 90% which is in good

comparison with published methods (*Kirkham, 1996; Aizwa et al., 1977*) where final product yields were between 80-90%.

The synthesised spiropyran dye was identified by performing a TLC analysis of the reagent products and synthesised solid. The results indicated at the purity of the synthesised product, further element analysis and melting point results were all in agreement to the purity and complete synthesis of the desired end product. An NMR analysis was unable to be performed on the sample. This would have confirmed the synthesised compounds structure and true identification.

The dye was characterised in ethanol, methanol and MES buffer (pH 6.8). It was seen that reverser photo-modulation occurred in aqueous solution or when attached to a protein. The reverse photo-modulation of the spiropyran dye in aqueous media, had also been published by *Namba et al (Namba et al., 1975)*.

5.2.3.b Photo-modulation of bioactive proteins

The aim of this study was to be able to photo-modulated Estradiol antibodies in order in apply them to the portable remote Spreeta™ analyser for the detection of endocrine disrupters in waste-water. Because of the limited supply of antibody horseradish peroxidase and anti -FITC were used to demonstrate the principle of photo-modulation. These two protein have also been studied by *Weston (Weston, 1999)*. Horseradish peroxidase is a robust protein that is extensively used in immunoassays and biosensors development. *Weston (Weston, 1999)* saw a decreased in activity by 53% due to covalent attachment of the dye to HRP, he suggested that due to dye attachment enzyme activity was inhibited. However, the results obtained in this study showed an increase in the HRP specific activity due to covalent attachment of the dye, both in solution and immobilized. The increase in HRP activity has not been reported before.. In a recent review publication on the stability of peroxidases, *Azverdo et al. (Azverdo et al., 2001)* noted that higher activities of HRP can be obtained when immobilised to a solid support, than when free in solution. This suggests that covalent attachment of HRP onto a solid support was able to suppress to some extent the unfolding process of the protein, conserving the active site and stabilising the enzyme (*Azverdo et al., 2001; Kirkham, 1996*). Therefore, the increase in enzyme activity seen in this study could be due to the dye having a similar effect. Where the conjugated spiropyran dye is influencing the enzyme bone structure in such a way that it conserves the integrity of the enzyme structure and affecting the substrate turn over rate. The activity rate of the modified HRP enzyme increased further when exposed to UV this must be due to an opening effect caused by the presence of the highly conjugated

merocyanine form. Being that the conjugated form may play a role in moving electrons in the enzyme substrate system and therefore increase the ability of the enzyme turnover activity. This finding would be of high value to affinity based systems where amplification of the detection signal is required and/or enzyme stabilisation is required.

5.2.3.c Towards the development of a photo-modulated Immunoassay

The initial study towards an immunoassay implementing photosensitive antibodies presented in this thesis indicated that regeneration of the bio-sensing surfaces by photomodulation is possible. Further investigation in this line enquiry would be required to confirm this position. Previous studies on photo reversible immunoassay by attaching photosensitive molecules to the antigen has been reported (*Kagner et al., 1999; Blonder et al., 1997; Kirkham, 1996; Harada et al., 1994*). The photochromic dye in this report was covalent attached using the water soluble EDC/NHS reaction. This reaction is not site specific and had a more site specific reaction would have been used (e.g. Sulfhydryl reaction) which targets sites on the antibody that was close to the active sites an increase the photomodulation affect of the antibody binding capability may have been seen.

Photo chromic dyes have been demonstrated to photo-modulate a wide range of bioactive proteins. Recent work by (*Muranaka et al., 2002; Hohsaka et al., 2002*) have shown that transcription of non natural photo-chromic moieties into bioactive protein structures is possible. This is a very interesting field as enzymes systems that could be modified and produced in such a way would open the field to a plethora of application. Photo-chromic dye modulation of bioactive proteins has been in the scientific arena for over thirty years and there is still a vast amount of investigation to be covered to build up enough momentum to advance in this course of study for true applications. It would be safe to speculate that incorporation of photo-chromic moieties in antibody systems is a complex phenomenon as there are many different interactions that may occur between antibodies and dyes. The application and interaction mechanism of non-natural moieties on the antibody structure is a very interesting area and one that has not been fully explored. Photosensitive enzyme system is also an interesting field were enzyme modulation on a biosensor system could help increase resolved signal output in electrochemical sensors and stabilise and preserve the enzyme for extended use plus, the added value of being able to change its rate of substrate turnover by a reagent-less photo control procedure. This would of course be of great use in enzyme labelled system where increased signal is need for detection at low concentration.

5.3 Future Work

5.3.1 Spreeta™ Sensor

The Spreeta™ sensor has been successfully demonstrated in detecting spiked water samples. It would then naturally follow for the sensor to be used with real samples in the field. For this to be realised a sample pre-concentration protocol step would need to be developed in order to clean and concentrate the environmental sample. Applying the sensor for the detection of other potential EDC by employing an estrogen receptor or polyclonal antibodies would also be a natural progression of this study. Further work on addressing surface modification and liquid handling atomisation is also required.

5.3.2 Photo-chromic dyes.

Further development and investigation into using photo-modulated antibodies for immunoassay systems would increase the knowledge and application of surface regeneration for biosensor applications. A more site specific method of covalently attaching the dye to the antibody and other bioactive proteins would be an appropriate course of study as optimisation of covalent dye attachment may increase the level in which the protein is photo-modulated. An extended investigation of photosensitised HRP in immunoassays and electrochemical detection to ascertain whether the introduction of the dye to the enzyme backbone structure really does stabilise the protein, as well as using the enzyme increased activity for photo-modulating the sensing signal (i.e. colourimetrically or potentiometrically) would be an interesting line of study.

5.4 Conclusions

The study of direct optical immuno-sensing and its application for measuring steroidal EDCs for remote and field analysis has been both interesting and challenging. The investigation and application of this study has resulted in the following conclusions.

- The Spreeta™ sensor by Texas Instruments is a very stable and robust SPR system that is well suited for original custom manufactured equipment. Incorporation of the sensor into a field analyser provided a controlled and self contained environment for the sensor to be used in the field.
- The gold sensing surface can be easily converted into an immunosensor by physical adsorption of an antigen- conjugated protein onto the surface. This was demonstrated to have good reproducibility.
- Three immunoassays for 17- β -Estradiol have been developed. An ELISA plate assay that has a working range of (0.3-7 μ g/L), the Spreeta™ analyser (0.3-7 μ g/L) and the Biacore @3000 system at (0.1-10 μ g/L). An immunoassay for Estrone-3-Gulcuronide was also developed which has a working range of (1.0 – 10 μ g/L). To detect Estradiol in real samples a sample pre-concentration step is required as Estradiol is often found at ppt levels in the field,
- The three immunoassays were able to detect spiked samples of Estradiol in synthetic and real wastewater sample at sub ppb levels. The level of accuracy was very high and fell within the acceptable accuracy range.
- The photo-modulation of horseradish peroxidase in free solution and covalent attached on a solid support increased its activity level by 79% and 72% respectively. The free conjugated enzyme was tested eight months later and 90% activity was retained plus photomodulation was still possible
- Introduction of the spiropyran dye on two different antibodies displayed different photo modulation effects. Both antibodies demonstrated a level of photo-modulation however Estradiol showed an increased rate of binding to its antigen while anti-FITC showed no difference but its binding affinity to FITC was reduced upon illumination with different wavelength.

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Appendices

Presented posters, published paper and presentations

Appendices

Poster Presentations

“Assessment of the Spreeta Miniaturised SPR Sensor for the Detection of Endocrine Disrupters in Water” A.M. Sesay, T. Kaplan & D.C. Cullen, Poster Presentation 4th Workshop on: Biosensors and biological techniques in environmental analysis, Mao Menorca, December 1-3, 1999.

“Use of a Commercial Miniaturised SPR system for the Detection of a Model Estrogenic compound” A.M. Sesay & D.C. Cullen, Poster presentation at 6th World congress on Biosensors (Biosensors 2000), San Diego, USA, 24-26 May 2000.

Published Paper

“Detection of hormone mimics in water using miniaturised SPR sensor” A.M. Sesay & D.C. Cullen, *Environmental Monitoring and Assessment*, **70**: 83-92, 2001, Kluwer Academic Publishers.

Presentations

“Spreeta™ SPR-based analyser for detection of Estrogenic compounds”, Seventh World Congress on Biosensors, Koyoto 15-17 May 2002

BIOSET workshop: Biosensors for evaluation of the performance of wastewater treatment works. Barcelona, April 5-7th 2000

Assessment of the Spreeta™ Miniaturised SPR Sensor for Detection of Endocrine Disrupters in Water

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Introduction

Endocrine disrupting compounds (EDCs) are a group of compounds that pose a potential threat to human and wildlife health by mimicking the biological effect of natural endogenous signalling chemicals, *e.g.* sex hormones. Thus, there is a desire for on-line or at-line analytical systems such as immunosensors for the monitoring of EDC levels.

We report the use of an “off-the-shelf” miniaturised integrated surface plasmon resonance (SPR) sensor to demonstrate its suitability for analysing estrogenic compounds in aqueous samples. A competition/inhibition assay has been developed involving a simple sensor immobilisation and regeneration scheme comprising physical adsorption of reagents and their subsequent removal via the novel use of a domestic laundry detergent. A re-useable sensor is demonstrated using estrone-3-glucuronide (E3G) as a model EDC and an anti-E3G antibody and producing a current working range between < 250 and 1000 ng/ml.

Sensor Concept & Design

To sensitively detect low molecular weight EDC analytes in an SPR sensor assay, amplification of the direct refractive index (RI) change is required. Therefore, we incubate samples with a soluble anti-EDC antibody prior to passing over the SPR sensor with immobilised antigen – *i.e.* detecting inhibition of antibody binding due to presence of appropriate EDC (~500 fold amplification compared to direct sensing).

To enable sensor reusability, we immobilise by physical adsorption a protein-EDC conjugate to the sensor surface for the inhibition assay format and then remove the all the protein/molecular assay components from the sensor surface with a novel washing step using domestic laundry detergents prior to a new sensor assay cycle.

Experimental Conditions

Surface Plasmon Resonance Instrument



Spreeta™ SPR sensors plus associated control box and software were used (Texas Instruments, USA). The Spreeta™ flow-cell was connected to a peristaltic pump (typical flow rate 10µl/min). Software settings: every 10 SPR scans averaged, data interval 2.5secs and SPR minima location by First Moment analysis.

Figure 1: Texas Instruments Spreeta™ SPR sensor.

Buffers and Reagents

REGENERATION BUFFER used to clean and regenerate the gold sensing surface – 1% domestic laundry detergent (Persil Biological Colour, Lever Brothers Ltd.) in Analar Water. REAGENTS: anti-E3G monoclonal IgG (clone 4155) and E3G-ovalbumin conjugate all in phosphate-buffered saline (pH 7.4).

Typical Sensor Analysis Cycle – see Figure 2

- Sensor surface cleaned/regenerated with flow of REGENERATION BUFFER for 10 mins then washed with water flow for 15 mins.
- 100µl of E3G-ovalbumin conjugate (150µg/ml) flowed passed cleaned bare gold sensor surface resulting in immobilisation of E3G by physical adsorption of conjugate.
- Anti-E3G IgG (100µg/ml) pre-incubated with known/unknown concentrations of E3G in water for 45 mins (un-optimised).
- 100µl of preceding assay mixture flowed passed immobilised E3G – amount of refractive index change/anti-E3G IgG bound recorded.
- Cycle repeated for next measurement

Novel Sensor Regeneration

Current “washing/regeneration” of affinity sensor surfaces commonly involves single detergents, variation of pH, *etc.* We have used domestic laundry detergent as an alternative – these contain complex mixtures of surfactants, proteinases, cellulases, lipases, *etc.* compared to simple traditional “recipes”.

Figure 2 shows a typical assay and regeneration cycle with an initial baseline in water. After adsorption of E3G-ovalbumin conjugate and subsequent antibody, the introduction of 1% Persil solution efficiently removed the adsorbed protein. This proved more reproducible and efficient than other washes such as 1M NaOH and 1% Triton X100 that required a longer washing step to achieve a comparable baseline.

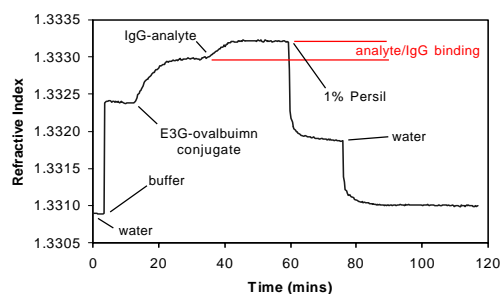


Figure 2: Typical sensor assay cycle demonstrating both sensor regeneration with domestic laundry detergent and measurement of antibody binding.

Sensor Assay Results

Figure 3 shows a calibration curve for the SPR sensor assay for E3G with the RI difference before and after the flow of the antibody-sample solution over the immobilised E3G layer. From this preliminary data, an initial working range between less than 250ppb and 1000ppb E3G can be estimated. A degree of non-specific binding of antibody can be seen.

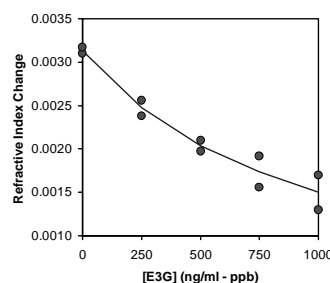


Figure 3: Calibration curve for the SPR sensor assay for E3G.

Discussion

The current sensor assay is un-optimised. The assay cycle time is excessive with significant reductions possible by modification of (i) flow-cell fluidics and (ii) length of sample-antibody pre-incubation. Whilst a model EDC has been used, the incorporation of endocrine receptors as affinity molecules should increase the spectrum of EDCs detectable. Detection limits need to be lowered via (i) optimised pre-incubation conditions and (ii) online solid-phase extraction steps to pre-concentrate the analytes.

Conclusions

- Demonstrated a simple reusable EDC inhibition/competition sensor concept using an “off-the-shelf” miniaturised SPR device and a model EDC analyte (estrone-3-glucuronide) and complementary antibody for sub-ppm detection.
- Demonstrated a novel sensor regeneration protocol using a domestic laundry detergent – allows simple regeneration of bare gold sensing surface involving reagent immobilisation via physical adsorption.
- Future work: (i) optimisation of current protocol and reagents, (ii) introduction of alternative receptors and analytes and (iii) automation of fluidics with online solid phase extraction/pre-concentration/sample clean-up.

Acknowledgements

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Use of a Commercial Miniaturised SPR System for Detection of a Model Estrogenic Compound

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Introduction

The widespread presence of chemicals in the environment with the capacity to disrupt the endocrine system in both wildlife and humans has in the last decade become an increasingly major concern. Endocrine disrupting compounds (EDCs) can mimic or interfere with the biological effect of natural endogenous signalling chemicals controlling the endocrine system. EDCs are prevalent in surface and waste-waters and thus there is a need for an at-source or at-line analytical systems such as biosensors for the monitoring of EDC levels.

We have incorporated an “off-the-shelf” miniaturised Texas Instruments Spreeta™ surface plasmon resonance (SPR) sensor into a field analyser and developed an competition/inhibition assay for a model estrogenic compound in aqueous samples that has the potential for *in-situ*, semi-continuous analysis of EDCs. A regeneration scheme employing the use of a domestic laundry detergent was used to strip and clean the sensing surface from absorbed protein. The re-useable sensor has been demonstrated using estrone-3-glucuronide (E3G) as a model EDC and an anti-E3G antibody producing a current working range of 0.075µg/ml to 0.5µg/ml.

Sensor Analyser

Surface Plasmon Resonance Instrument

Spreeta™ SPR sensors plus associated control box and software were used (Texas Instruments, USA). The Spreeta™ flow-cell was connected to a manual sample loop injection valve. Reagent buffers were pulled through the flow cell using a peristaltic pump (typical flow rate 10µl/min) (see Figures 1 and 2). Software settings: every 20 SPR scans averaged, data interval 5secs and SPR minima location by First Moment analysis.



Figure 1: External view of field analyser

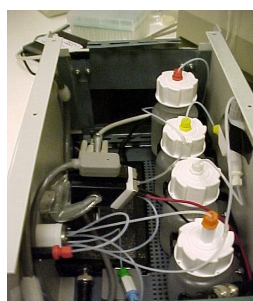


Figure 2: Internal view showing sensor and reagent reservoirs

Experimental Conditions and Assay Design

Reusability of the sensor was achieved by *in-situ* immobilisation by physical adsorption of a protein-EDC conjugate to the sensor surface for a subsequent inhibition assay with soluble antibody. 150µl of an ovalbumin-E3G conjugate was flowed passed the sensing surface to physical adsorb and immobilise the competing analyte.

Total removal of all the protein/molecular assay components from the sensor surface was reproducibly achieved by employing a surface regeneration step using a domestic “biological” laundry detergent prior to a new sensor assay cycle. *Buffers and Reagents*

REGENERATION BUFFER: used to clean and regenerate the gold sensing surface – 1% domestic laundry detergent (Persil Biological, Lever Brothers Ltd., UK) in Analar water and filtered using 0.2µm filter. **RUNNING BUFFER:** phosphate buffer saline (pH 7.4). **CALIBRATION WATER:** Analar water. **REAGENTS:** anti-E3G monoclonal IgG (clone 4155) at 100µg/ml and ovalbumin-E3G conjugate at 150µg/ml all in phosphate-buffered saline (pH7.4). All solutions were de-gassed.

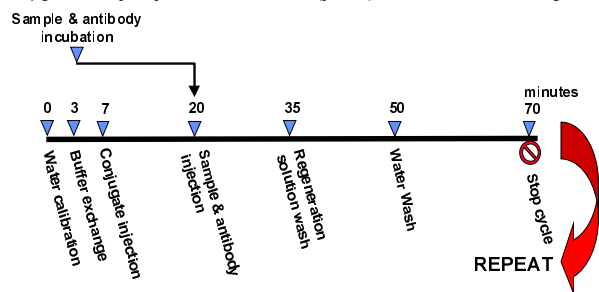


Figure 3: Typical sensor assay cycle demonstrating both sensor regeneration with domestic laundry detergent and measurement of antibody binding.

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Results

Surface Regeneration and Conjugate Baseline Results

Current “washing/regeneration” of affinity sensor surfaces commonly involves single detergents, variation of pH, *etc*. By using a domestic laundry detergent as an alternative (containing complex mixtures of surfactants, bleaching agents, proteases, cellulases, lipases, *etc*) we were able to achieve a reproducible baseline and subsequent conjugate adsorption for repeated assays (see Table 1).

Regeneration with “Persil”	SPR Resonance Angle (°)
Water baseline	69.158 ± 0.164
PBS baseline	69.340 ± 0.063
Conjugate baseline	69.356 ± 0.045

Table 1: Effectiveness of sensor regeneration using a commercial laundry detergent - number of repeated cycles = 15.

Non specific binding studies have been carried out by flowing passed unrelated antibody across the immobilised conjugate layer with no significant change in baseline observed.

Sensor Assay Results

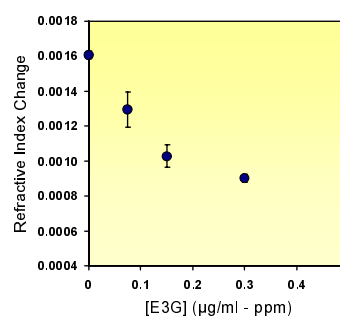


Figure 4 shows a calibration curve for the SPR sensor assay for E3G with the RI difference before and after the flow of the antibody-sample solution over the immobilised E3G layer. From this preliminary data, an initial working range between less than 75ppb and 500ppb E3G can be estimated.

Figure 4: Calibration curve for the SPR sensor assay for E3G.

Discussion and Future Work

The current sensor assay is un-optimised. The current assay cycle time is excessive with significant reductions possible by modification of (i) flow-cell fluidics and (ii) length of sample-antibody pre-incubation. Whilst a model EDC has been used, the incorporation of endocrine receptors as affinity molecules should increase the spectrum of EDCs detectable. Detection limits need to be lowered via (i) optimised pre-incubation conditions and (ii) on-line solid-phase extraction steps to pre-concentrate analytes and reduce potential non-specific effects.

Conclusions

- Established the incorporation of an “off-the-shelf” miniaturised SPR device into a field analyser that can be used with a simple inhibition binding assay for a model EDC analyte (estrone-3-glucuronide) and complementary antibody for sub-ppm detection.
- Reusability of sensor was achieved by using a domestic laundry detergent – allowing regeneration of bare gold sensing surface and reproducible protein immobilisation via physical adsorption.

Acknowledgements

This research has been partially undertaken as a component of the SANDRINE project (Project No. ENV4-CT98-0801) funded by the DG XII Environment and Climate (1994-8) work programme of The European Commission.

We also thank Drs A. Badley & I. Jonrup of Unilever Research, Bedford, UK for supply of antibodies and conjugates.

DETECTION OF HORMONE MIMICS IN WATER USING A MINITURISED SPR SENSOR

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Abstract. The ubiquitous presence of chemicals, both natural and synthetic, in the environment with the potential to mimic hormones that may in turn interfere with the endocrine system in both wildlife and humans has in the last decade become a major international concern. Hormone mimics or endocrine disrupting compounds (EDCs) are especially prevalent in surface and waste-waters and therefore, there is a need for an at-source or at-line analytical device for the monitoring of EDC levels. We have incorporated a miniature integrated surface plasmon resonance (SPR) liquid sensor from Texas Instruments into a field analyser and developed a competition/inhibition assay for a model estrogenic compound in aqueous samples. The analyser has the potential for *in situ* and semi-continuous analysis of EDCs. A novel regeneration scheme employing the use of a domestic laundry detergent has been used to remove immobilised assay components between each assay cycle. The resultant re-usable sensor has been demonstrated using estrone-3-glucuronide (E3G) as a model EDC and an anti-E3G antibody producing a current detection range of 10 to 150 ng mL⁻¹.

Keywords: endocrine disrupting compounds, estrone-3-glucuronide, hormone mimics, miniturised SPR sensor, surface plasmon resonance

1. Introduction

Hormones are biologically active substances that are secreted into the blood system via ductless glands of the endocrine system. They are active at very low concentrations (ng mL⁻¹ to pg mL⁻¹, i.e. ppb or ppt) and bind specifically to target receptor sites on cell surfaces or within the cell nucleus. Once associated with their corresponding target site they exert important regulatory, growth, homeostatic or reproductive effects. The complexity of the endocrine system with cascading loops of hormone signals and responses lends it self to the interference of the system at many points (Arnold and McLachlan, 1996; US EPA, 1997) and hence sensitivity to environmental natural and non-natural hormones and hormone-mimics, i.e. endocrine disrupting compounds.

The purpose of the work reported here is to provide an initial demonstration of the application of a portable biosensor or bioanalyser device that could be implemented near or at source (i.e. wastewater sewage treatment works or surface waters) to determine concentration levels of hormones and their mimics in aqueous environments. It is the aim of this on-going study to achieve this via a simple,



sensitive and stable surface bio/chemistry for the biosensor that can be replaced remotely and automatically by a simple combination of fluidics, non-covalent immobilisation and cleaning steps. Therefore for immobilisation, we have adopted the physical adsorption from aqueous solution to the gold sensor surface of a carrier protein-EDC conjugate to immobilise the specific analyte/hapten for a subsequent competition/inhibition assay. A competition/inhibition assay was chosen to enable the sensitive detection of low molecular weight EDC analytes/haptens in a SPR sensor assay. The assay comprises the pre-incubation of samples with a soluble anti-EDC IgG antibody prior to passing over the SPR sensor with immobilised analyte/hapten. By detecting inhibition of antibody binding due to the presence in a sample of an unknown concentration of an appropriate EDC, a potential 500 fold amplification compared to direct sensing of the low molecular weight EDC analyte/hapten can be expected.

To enable sensor reusability, a novel regeneration step using a domestic liquid laundry detergent was used to achieve reproducible removal of all protein and other molecular assay components from the sensor surface, i.e. regenerating a clean gold surface layer prior to each sensor assay cycle.

2. Materials and Methods

2.1. INSTRUMENTATION – SENSOR ANALYSER

The experiments were carried out using the Spreeta™ evaluation Module Kit manufactured by Texas Instruments Inc. (TI) (Texas, U.S.A.) (Woodbury *et al.*, 1998; Elkind *et al.*, 1999; Kukanskis *et al.*, 1999). The commercially available package consists of 50 miniature SPR sensors, associated electronic control box, flow-cell and software. The Spreeta™ sensor is a fully integrated device where all the components required for SPR such as light source and detector are integrated on a small chip and encapsulated in an optical clear epoxy element to enable a standard ‘wedge-beam’ Kretschmann prism SPR type arrangement that requires no further optical alignment after manufacture (see Figure 1).

The Spreeta™ SPR sensor has been integrated into a self-contained analyser (see Figure 2) and is comprised of a steel housing containing the Spreeta™ SPR device, TI flow-cell, TI control electronics, manual sample loop injection valve and liquid switching valve (Ominfit, Cambridge, U.K.) and reagent reservoir bottles. Reagents and buffers are pulled through the flow-cell using a peristaltic pump (at present using an external pump – Minipuls 3, Gilson, U.K.) at a typical flow rate of $60 \mu\text{L min}^{-1}$.

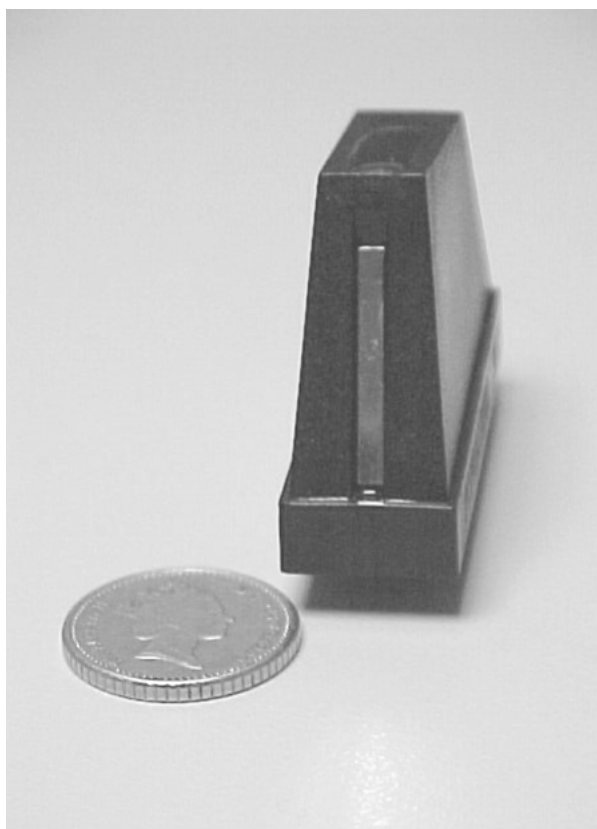


Figure 1a. The Texas Instrument Spreeta™ sensor showing gold sensing surface and encapsulation.

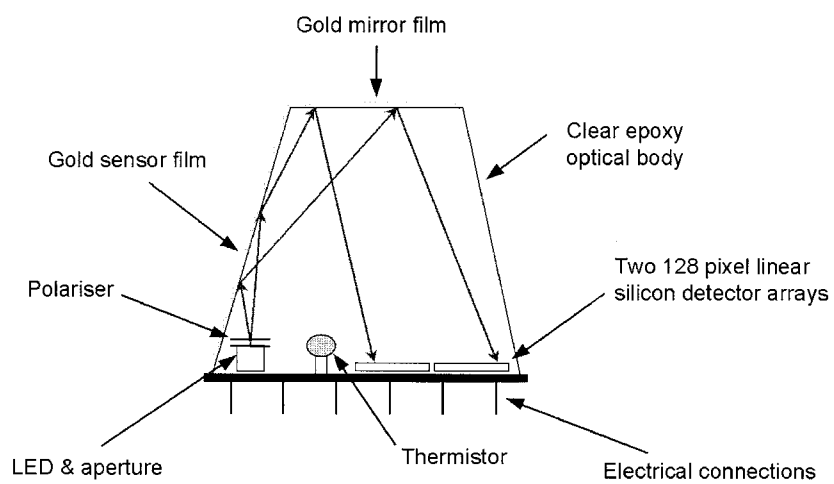


Figure 1b. Schematic of internal structure of an early Texas Instrument SPR sensor—sensing region is labelled ‘gold sensor film’.

2.2. SOFTWARE SETUP

The Spreeta™ SPR system is supplied with dedicated control and data handling software and was run on a notebook personal computer associated with the bioanalyser (see Figure 2). Typical software parameters used for experimentation were as follows:

- Number of automated measurements set to 20 (therefore SPR data output over 20 recorded data event values averaged to make 1 datapoint);
- Minimal monitoring interval (time required to drive the sensor and analyse the result) set to 0.25 sec (i.e. $20 \times 0.25 = 5$ sec per recorded datapoint);
- Every other data point event recorded (i.e. 10 sec between each saved data value);
- First Moment used as analysis method to determine SPR minimum position and hence refractive index.

2.3. BUFFERS AND REAGENTS

Phosphate buffered saline, pH 7.4 (PBS) (Sigma, U.K.) used as a running buffer to prime the sensor surface and for rinsing and Analar Water (BDH, Poole, U.K.) used for buffers and for refractive index calibration. The domestic laundry detergent used as a surface Regeneration Buffer was 1% (v/v) Persil Biological Liquid (Unilever, U.K.) in water.

Anti-Estrone-3-Gulcronide antibody (monoclonal IgG clone 4155, a kind gift provided by Unilever Research plc. U.K.) was made up to $200 \mu\text{g mL}^{-1}$ in PBS and 0.05% Tween 20.

Calibration samples of E3G (Sigma, U.K.) were prepared by dissolving 1 mg of E3G in 1 mL of dimethylformamide (DMF) and then making a stock solution of E3G at $1 \mu\text{g mL}^{-1}$ with PBS. Ovalbumin-E3G conjugate was supplied by Unilever Research plc (U.K.) at $150 \mu\text{g mL}^{-1}$ made up in PBS. All solutions prior to use were degassed under vacuum at room temperature to minimise bubble formation in the fluidic system.

2.4. PROCEDURES

The gold surface of the sensor was cleaned prior to performing an assay with the Regeneration Buffer for 10 min and then washed with water for a further 10 min.

This was important especially when using a new sensor or after extended dry storage. Once washed the sensor was then dried with a nitrogen gas stream prior to performing sensor initialisation. A typical procedure for performing an assay cycle follows:

- Before commencement of an experiment the sensor was initialised in air and then calibrated in water to establish a background reading were all following measurements would be referenced to;



Figure 2a. External view of field-analyser showing external peristaltic pump and control notebook computer.

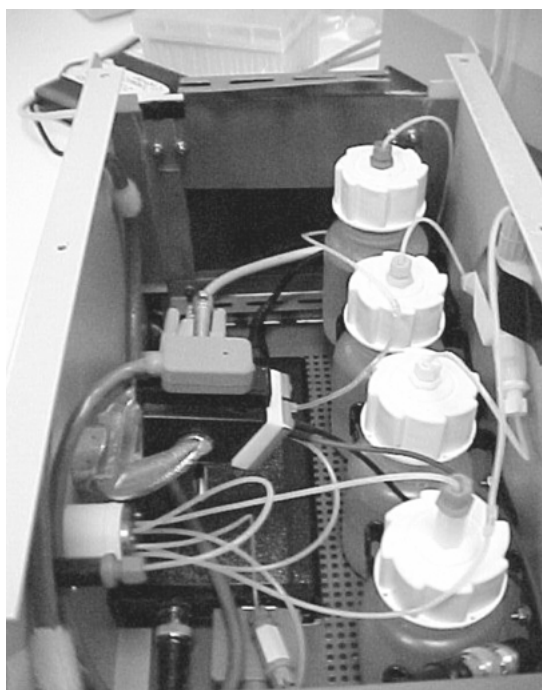


Figure 2b. Internal view showing sensor and flow-cell connected to sample valve and reagent bottles.

- PBS running buffer was flown past the surface for 5 min to obtain a baseline;
- Ovalbumin-E3G conjugate (200 μL) was injected in to the sample loop and then allowed to pass over the sensor surface after a PBS baseline was established;
- PBS was allowed to wash the sensor for 10–15 min to remove any loosely bound protein conjugate and obtain adsorbed conjugate only baseline measurement;
- An equal volume of anti-E3G IgG (100 $\mu\text{g mL}^{-1}$) was pre-incubated with sample (known/unknown concentrations of E3G) for 30 min;
- 200 μL of preceding assay mixture was then injected and allowed to flow passed the sensor surface with immobilised protein conjugate;
- PBS again washed over the sensor for 10–15 min to remove any loosely bound antibody and obtain a baseline measurement;
- The sensor surface was then cleaned/regenerated by flowing past the regeneration buffer over the gold surface for 15 min and then rinsing the surface with water for 10 min.

Once the assay procedure was completed the cycle was repeated for next sample measurement. Air initialisation and calibrating in water was not a required step for subsequent measurements.

2.5. SPR DETECTION AND DATA ANALYSIS

The automatically recorded data according to the parameters set, were viewed from the data table and transferred directly in to a Microsoft Excel 97 spreadsheet. The time verse refractive index or angle sensorgram scan obtained was then analysed manually to obtain changes in angle over time and relevant information on signal to noise data.

3. Results and Discussion

3.1. INTERFACE REGENERATION

One of the main aims of this on-going research is to provide a practical and reproducible affinity assay to be used with the SpreetaTM liquid SPR sensor. As we have considered the gold sensing surface to be practically irreplaceable, our main objective was to use the SpreetaTM sensor as a biosensor and attach a biologically specific layer on the sensing surface and in turn find a regeneration protocol that would be able to strip the surface of adsorbed protein thereby enabling the sensor to be reused. Typically ‘washing’ or ‘regeneration’ procedures for affinity sensor surfaces commonly involves exposure to single component detergents, variations of pH, variations of ionic strength, etc. We have employed a domestic laundry

Regeneration with Persil	SPR Resonance Angle (°)
Water Baseline	69.158 ± 0.164
PBS Baseline	69.340 ± 0.063
Conjugate Baseline (in PBS)	69.356 ± 0.045

Figure 3. Demonstration of repeated regeneration of a gold SPR sensor surface after physical adsorption of protein using a commercial laundry detergent (± 1 SD, $n = 15$).

detergent as an alternative that contains a defined complex mixture of surfactants, proteases, cellulases, lipases and bleaching agents compared to simple traditional regeneration approaches.

Figure 3 summarises the effect on the optimum incident angle for SPR excitation for repeated immobilisation of E3G-ovalbumin conjugate and regeneration of the bare gold surface via the domestic laundry detergent. The average SPR angle increases as expected from water to PBS to conjugate as the refractive index experienced by the surface plasmons increases. The small average increase due to the adsorption of the E3G-ovalbumin conjugate is due to the de-naturation of the protein upon adsorption. Since the functionality of the conjugate is due to the availability of the E3G, the loss of the native ovalbumin structure is of no direct consequence. The variation of the SPR angle for the various steps in the 15 repeats included in Figure 3, demonstrate a significant level of variability in the absolute SPR angle value though the relative changes within cycles are not as significant.

3.2. SENSOR ASSAY RESULTS

The second aim of this current report was to demonstrate that the Spreeta™ sensor could be used as a biosensor to determine low molecular weight analytes such as EDC's for the eventual purpose of using the instrument as an automated field-analyser. Although the current competition/inhibition assay is not optimised, we have demonstrated a calibration curve for the model EDC, E3G, with good reproducibility for a current working range between 10 and 150 ppb (see Figure 4). This situation compares with published microtitre-plate based receptor assays with lower limits of detection of 0.1 ppb ($0.1 \mu\text{g L}^{-1}$) for the related 17β -estradiol (Seifert *et al.*, 1999). For the current study, E3G was chosen as the model EDC due to the ready availability of appropriate antibodies within our laboratories. The SPR data for an example single assay cycle is also shown in Figure 5.

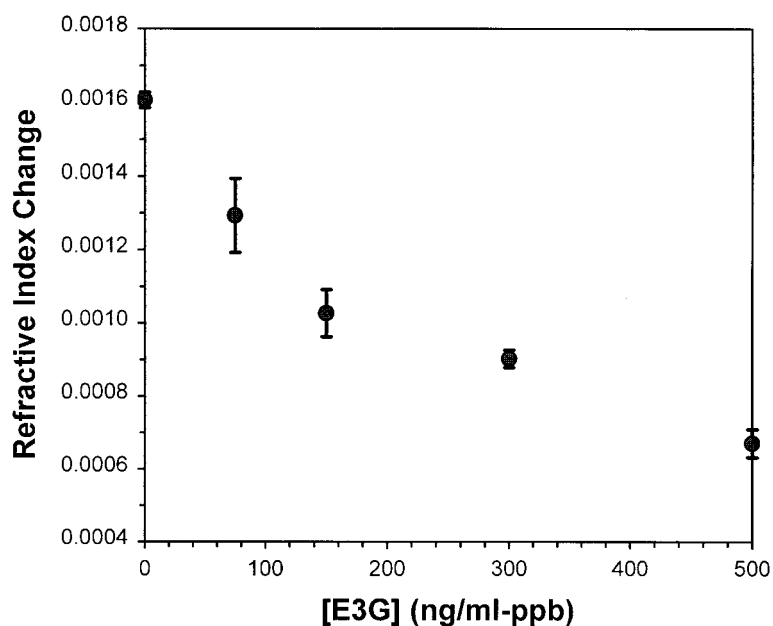


Figure 4. Calibration curve for the SPR sensor assay for E3G with the RI difference before and after the flow of the antibody-sample solution over the immobilised E3G layer. From this preliminary data, an approximate working range between 10 and 150 ppb E3G can be estimated (error bars are ± 1 SD; $n = 3$).

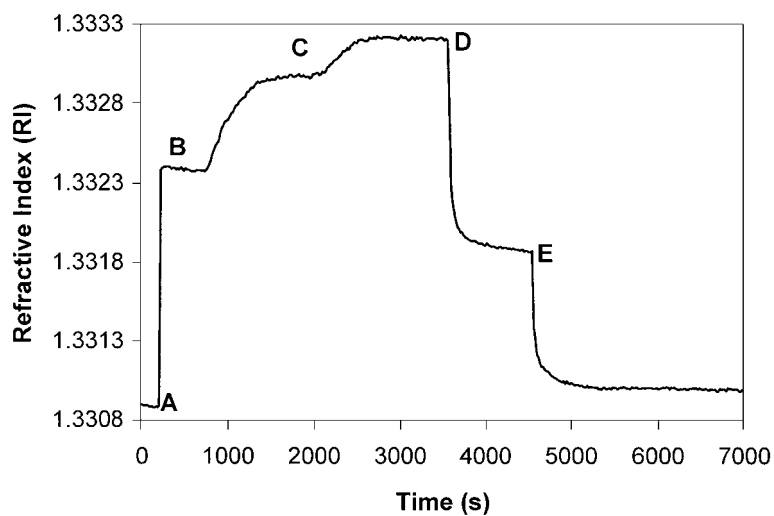


Figure 5. A typical assay time trace where A is the baseline of water, B is the baseline of phosphate buffer saline solution 7.4 pH, C is the addition and binding of anti-E3G, in the present of competing E3G, to E3G-ovalbumin conjugate, D is the regeneration of the gold surface using 1% Persil solution and E is the final rinsing with water. The amount of antibody binding is found by determining the refractive index change between conjugate baseline and antibody baseline (difference between immediately prior to point C and prior to point D).

The procedure for each assay cycle is as described in the Materials and Methods section. Figure 5 shows a typical SPR output trace of such an assay cycle and shows the various phases of the cycle and including the immobilisation of the ovalbumin-E3G conjugate, the binding of the anti-E3G antibody and the regeneration of gold sensing surface.

3.3. FURTHER DEVELOPMENTS

Further refinement and optimisation of the current assay is required as time-per-assay-cycle, reduction of reagent (i.e. antibody) consumption and detection levels needs to be addressed. The time for a single assay cycle is currently excessive as it takes approximately 70 min for a full cycle to be completed. Significant reductions are expected by modification of the flow-cell fluidics and increased automation. By reducing the flow-cell volume and increasing the flow rate we will be able to improve the kinetics and mass transfer of solutes flowing passed the sensing surface while reducing the amount of reagents used. Additionally, the length of sample-antibody pre-incubation has not been optimised.

The current detection limits demonstrated needs to be lowered if the sensor is to be used in the field as levels of natural oestrogen are usually in the parts-per-trillion range. The use of a pre-concentration step, i.e. solid-phase extraction or an affinity column, will help to address detection limits and to reduce potential matrix effects present in real samples. Furthermore, to broaden the range of EDC's detectable and eventually have a true EDC sensor, the incorporation of recombinant endocrine receptors as affinity molecules can be envisaged.

4. Conclusions

There is a growing need for the analysis of potential environmental contaminants to be conducted at or near source and in real-time. Therefore, the need for portable, simple, low cost devices is of great importance. As hormone mimics are especially prevalent in surface and waste-water, our aim was to investigate whether the Spreeta™ SPR sensor was an applicable device to be used as a portable bioanalyser.

The current status of this on-going project provides evidence that the 'off-the-shelf' miniaturised SPR Spreeta™ liquid sensor can be incorporated into a field analyser and can be used with a simply assay protocol for a model EDC analyte (estrone-3-glucuronide) using a complementary antibody for sub-ppm detection. The desired reusability of the sensor was achieved by employing a novel approach using a domestic laundry detergent allowing the regeneration of the bare gold sensing surface and reproducible protein conjugate immobilisation via physical adsorption.

Future developments of this approach will include, (i) optimisation of assay protocols, (ii) improved fluidics, (iii) integration into the analyser of pumps and auto-

mated sampling/fluid switching and (iv) up-stream sample pre-treatment including filtering and solid-phase extraction/pre-concentration.

Acknowledgements

This research has been undertaken as a component of the SANDRINE project (ENV4-CT98-0801) funded by the DG XII Environment and Climate (1994–1998) work programme of the European commission. We also thank Drs. A. Badley and I. Jonrup of Unilever Research, Bedford, U.K. for supply of antibodies and conjugates. This paper was presented at the 3rd International Symposium in Advanced Environmental Monitoring, Cheju Island, Korea, 31 October–2 November 2000. Part of the research was performed at the EC BIOSET Technical Meeting–Biosensors for Evaluation of the Performance of Waste Water Treatment Works, Barcelona, Spain, 5–7 April 2000 and presented in the report of this meeting.

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