CRANFIELD UNIVERSITY

CRANFIELD HEALTH

PhD THESIS

ACADEMIC YEAR 2009-2010

DIMITRIS KYPRIANOU

DEVELOPMENT OF NOVEL MATRICES FOR BIOMOLECULE IMMOBILISATION ON SENSOR SURFACES

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This thesis is submitted in partial fulfillment of the requirements

for the degree of Doctor of Philosophy

Abstract

The development of a novel protocol for the covalent immobilisation of biomolecules containing primary amines using either polythiol compounds or novel, inexpensive and simple polymers is presented in this thesis. When developing biosensors, the method used for the immobilisation of the sensing elements is very important. The immobilisation needs to be fast, cheap and most importantly should not affect the biorecognition activity of the immobilised receptor. The chemistry used for the immobilisation is based on the well known reaction between primary amines and thioacetal groups, formed upon reaction of o-phthaldialdehyde (OPA) and thiol compounds. Initially the possibility to use this chemistry to immobilise receptors and develop biosensors was proved using commercially available polythiol compounds. Such compounds can be irreversibly adsorbed, creating self-assembling monolayers (SAMs), on noble metal transducer surfaces. These SAMs were immobilised on Biacore surface plasmon resonance (SPR) gold chips and then used to study kinetic of biomolecules interactions and to detect cells. A general protocol suitable for the immobilisation of enzymes and antibodies such as anti-prostate specific antigen (anti-PSA) and anti-*Salmonella typhimurium* antibody was optimised. Kinetic data were obtained for PSA binding to anti-PSA antibody and they were compared to the results obtained using commercially available Biacore chips, CM1. For *Salmonella typhimurium* cells, a detection limit of 5×10^6 cells ml^{-1} with minimal non-specific binding of other biomolecules was obtained. An interesting capability shown by these SAMs, in contrast with commercially available chips, was the opportunity to immobilise any proteins, even those with very low or high isoelectric points, pI. In addition protein immobilisation was achieved with a simple step, without requirement of any activation. These findings make this immobilisation technique a very promising alternative to peptide bond formation for amine coupling.

Even though, the developed SAMs showed to be useful for certain type of applications (kinetic study and detection of very large analyte), it was clear that due to a combination of factors (e.g. limited and steric hindrance), they were not suitable for the development of biosensors good enough for practical applications. Therefore to overcome the drawbacks shown by polythiol SAMs, a novel 3-D polymer was developed. The main advantage of this polymer is the tridimensional (3D) network, which, after

immobilisation, ensures the availability of a high percentage of receptor binding sites. As the polythiol SAMs, also the 3-D polymer contains thioacetal groups, which do not need any activation to react with primary amines in proteins. The novel 3-D polymer also contains thiol derivative groups (disulphide groups or thioethers) that promote self-assembling on metal surfaces. As before, the polymer was immobilised on SPR gold chips and the resulting layer was characterised using contact angle meter, atomic force microscopy (AFM) and ellipsometry. Contact angle demonstrated that the immobilisation of polymer on sensor surface produced a relatively hydrophobic surface. The thickness of polymer layer was determined by applying ellipsometry, whereas AFM showed the change of surface roughness after polymer attachment. A general protocol suitable for the immobilisation of BSA, enzymes and antibodies such as polyclonal anti-microcystin-LR and monoclonal anti-prostate specific antigen (anti-PSA) antibody was then optimised. The affinity characteristics of developed immunosensors were investigated in reaction with microcystin-LR, and PSA. The calculated detection limit for analytes depended on the properties of the antibodies. The detection limit for microcystin-LR was 10 ng ml^{-1} and for PSA 0.05 ng ml^{-1}. The 3-D polymer chips were stored for up to 2 months without any noticeable deterioration in their ability to react with proteins. The performance of 3-D polymer chips were also compared with commercially available Biacore chips, as CM5. The main advantages were found to be the low cost, the possibility to immobilise biomolecules at physiological pH (pH 7.4), the lack of any activation step for biomolecules immobilisation and the opportunity to immobilise proteins with very different pI (also very low pI).

Despite the successful detection of PSA achieved in buffer (detection limit 0.05 ng ml^{-1}) using 3-D polymer chips, the detection of proteins in serum resulted to be very challenging due to the complex nature of the matrix, which contains a high content of many different compounds. Different techniques were applied in order to reduce the non specific adsorption of serum on 3-D polymer sensors with antibodies immobilised on the surface. Satisfactory results were finally obtained by including the surfactant P20 into the measuring system. The detection of PSA in serum using 3-D polymer sensors, however, became possible only by switching from a direct detection to a ‘sandwich detection’. In this sandwich format, after injecting samples of PSA (prepared both in buffer or 20%

serum) onto a specific antibody (capture-Ab, C-Ab) immobilised on the 3-D polymer surface, the analytical signal is recorded by injecting a second specific Ab (detection-Ab, prepared in PBS), which recognises a different epitope of the antigen. With this format, the analytical signal is recorded in absence of any complex matrix, avoiding interference from non specific adsorption. The detection limit for PSA, obtained using the sandwich immunosensor (developed on 3-D polymer chips) was 0.1 ng ml^{-1} in buffer and 5 ng ml^{-1} in 20% serum, which is very close to the sensitivity necessary for detection of the prostate biomarker in real samples. Therefore this study has demonstrated the opportunity to apply the novel 3-D polymer for development of biosensors suitable for applications in real samples.

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NOTATIONS

Symbols (unit):

^{o}C	Celcius
cm^2	Square centimetre, surface
Da	Daltons
fM	Femtomolar
g	Gram, mass
Hz	Hertz, frequency
l	Litre, Volume
J	Joule, energy unit
M	Molar, concentration
mg	Miligram
min	Minutes, time
ml	Millilitre
mM	Milimolar
MHz	MegaHertz
nm	Nanometre
nmol	Nanomole
pH	Acidity measurement unit
pM	Picomolar
ppb	Parts per billion

ppm	Parts per million
ppt	Parts per trillion
RU	Biacore Response Units
s	Second
w	Watt
µg	Microgram
µl	Microlitre
µm	Micrometre
µM	Micromolar

Abbreviations

3-D	3-Dimensional
Ab	Antibody
ACN	Acetonitrile
AFM	Atomic Force Microscopy
Ag/AgCl	Silver/Silver Chloride
Ag	Antigen
AIBN	Triethylamine, Azo –isobutyronitrile
Al	Aluminium
Al_2O_3	Aluminium Oxide
Ar	Argon
AT	Allyl thiol
Au	Gold
BAC	Bis(acryloyl)cystamine
Bap	2,4-D,-Benzo (a) pyrene
BDMB	2-benzyl-2 (dimethylamino)-4-morpholino-butyrophenone
BOD	Biochemical Oxygen Demand
BSA	Bovine Serum Albumin
CA	Contact Angle
Ca-Ab	Capture Antibody
CCD	Charged Coupled Device

CMCI	1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho p-toluene sulfonate
CV	Coefficient Variation
D-Ab	Detection Antibody
DAB	3,3´-diaminodbenzidine
DCC	1,3 – dicyclohexylcarbodiimide
DDT	DL-dithiothreitol
DMF	Dimethyl Formamide
DMTZ	2,5-dimercapto-1,3,4-thiadiazole
DNA	Deoxyribonucleic acid
DTNB	5,5'-dithio-bis(2-nitrobenzoic acid)
DTT	Dithiothreitol
EDC	1-ethyl-3-dimethyl (aminopropyl) carbodiimide
EGDMA	Ethylene glycol dimethacrylate
EDTA	Ethylenediamine Tetraacetic Acid
ELISA	Enzyme-linked immunosorbent assay
EMCH	N- [ε- maleimidocaproic acid]- hydrazide
ETA	Ethanolamine
EU	European Union
Fc	Fragment crystallisable
Fab	Fragment antigen binding
FBAR	Film bulk acoustic resonators
FDA	Food and Drug Administration

FET	Field Effect Transistor
FTIR	Frustrated Total Internal Reflection
GC	Grating Coupled
Ge	Germanium
GOD	Glucose Oxidase
GMO	Genetically Modified Organisms
GPC	Gel Permeation Chromatography
GR	Glutathione Reductase
GST	Glutathione- S-Transferase
HACCP	Hazard Analysis of Critical Control Points
HCl	Hydrochloric Acid
H_2O_2	Hydrogen peroxide
HEM	2- hydroxyethyl methacrylate
HEPES	4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid
HDT	1,6-hexanedithiol
His	Histidine
HOPG	Highly Orientated Pyrolytic Graphite
HPLC	High Performance Liquid Chromatography
HSA	Human Serum Albumin
IgG	Immunoglobulin G
IR	Infra Red
ISO	International Standard Organisation
IUPAC	International Union of Pure and Applied Chemistry

ka	Association constant
kd	Disassociation constant
LC	Liquid Chromatography
LD	Lipoamide dehydrogenase
LOD	Limit of Detection
mAb	Monoclonal antibody
Microcystin-LR	Microcystin-Leucine, Arginine
MIPs	Molecular Imprinted Polymers
Mn	Molecular weight averages
MMA	Methyl Methacrylate
Mn	Molecular number
MS	Mass Spectroscopy
Mw	Molecular Weight
N_2	Nitrogen
$Na_2B_4O_7$	Sodium Borate
NaCl	Sodium Chloride
NADH	Nicotinamide adenine dinucleotide reduced form
NADP	Nicotinamide adenine dinucleotide phosphate
NaH_2PO_4	Sodium dihydrogen phosphate
NaOH	Sodium Hydroxide
NDT	1,9-Nonanedithiol
NH_4OH	Ammonium Hydroxide
NHS	N-hydroxysuccinimide

NTA	Nitrilotriacetic acid
O_2	Oxygen
OPA	o-phthalic dialdehyde
PAMAM	Polyamidoamine
PANI	Polyaniline
PBS	Phosphate Buffered Saline
PCB	Polychlorobiphenyl
PCR	Polymerase Chain Reaction
PDI	Molecular weight distributions
PETMP	Pentaerythritol tetrakis (3-mercaptopropionate)
pI	Isoelectric point
POC	Point of Care
Pol.	polymer
PPy	Polypyrrole
PP	Polypropylene
PSA	Prostate Specific Antigen
PVC	Polyvinyl Chloride
QCM	Ouartz Crystal Microbalance
RF	Radio Frequency
RI	Refractive Index
RII	Refractive Index Increment
RNA	Ribonucleic Acid
ROMP	Ring Opening Metathesis Polymerisation

rpm	rotations per minute
RuO_2	Ruthenium Dioxide
SAM	Self -Assembled Monolayer
SAW	Surface Acoustic Wave
SCE	Saturated calomel electrode
SDS	Sodium Dodecyl Sulfate
Se	Selenium
SH	Thiol group
SiO_2	Silicon Dioxide
SGFT	Suspended Gate Field Transistors
SMR	Surface mounted resonators
SnO_2	Tin Oxide
SPR	Surface Plasmon Resonance
ss-DNA	Single Stranded-DNA
ST	*Salmonella Typhimurium*
STD	Standard deviation
Sulfo-GMBS	N-[γ- maleimidobutyryloxy] sulpho- succinimide ester
TCDD	2,3,7,8- tetrachlorodibenzo-p-dioxin
TEA	Triethylamine
THF	Tetrahydrofurane
TiO_2	Titanium Dioxide
TIR	Total Internal Reflection
TMPTMA	Trimethylolpropane tris (2-mercaptoacetate)

TNT	Trinitrotoluene
TRIM	1,1,1-tris (hydroxymethyl)propan trimethacrylate
UV/Vis	Ultra Violet/Visible
XPS	X-ray Photoelectron spectroscopy
Zn	Zinc

CHAPTER 1

INTRODUCTION- AIMS AND OBJECTIVES

1.1. Structure of Thesis

The work presented here starts with an introduction to biosensors covering history of biosensors and their current applications. A review of the research carried out in the field of surface plasmon resonance (SPR) and quartz crystal microbalance (QCM) sensing, reporting the main achievements, follows. After this, a description of the methods, described in the literature, used for sensor surface modifications and receptor immobilisation is presented. The methods for surface modification range from monolayers to electropolymerised films or hydrogels, whereas techniques used for immobilisation of biomolecules include physical adsorption, entrapment, high affinity biological reaction and covalent coupling. The experimental part of thesis follows and is divided in three chapters. In Chapter 3 the application of polythiols monomer layers for amino coupling through the reaction of thioacetals with primary amines resulting in formation of a fluorescent isoindole is described. Chapters 4 and 5 describe the development of novel polymers employing the same chemistry as the polythiol monomers for protein immobilisation and their application on SPR immunosensors. In particular, in Chapter 5 the novel polymer is applied for the development of a SPR immunosensor for prostate specific antigen (PSA) detection in buffer and serum. The last chapter includes some general conclusions and the future work, which should be carried out to bring the novel material, developed here, to mass production and commercialisation.

1.2. Introduction - Aims and Objectives

The market for immunoassay and immunosensor technology in areas such as food safety, drug discovery, environment and clinical analysis is set to grow, with a steadily increasing demand for cost-effective, sensitive and easy to use sensors

(Sadana, 2006). This is due to the fact that a successful biosensor reduce dramatically the time of analysis, there is a potential of miniaturisation and it is user friendly. There is no need for any special skills in order to perform analysis with established biosensor system. One particularly important area of biosensors is the development of point-of-care (POC) and label-free devices, with special emphasis on clinical assays for early cancer diagnostics (Sadana, 2006; Wang, 2006). A wide range of biosensor systems for real-time detection of nucleic acids and proteins have been developed in recent years, which potentially could be applied for medical diagnosis and individualised medicine in the future. A small number of these detection systems, however, have been commercialised. In fact due to their lack of usability, size and costs, these systems are only suitable for operation in highly specialised laboratories, making testing expensive and time-consuming. For this reason many of these new tests are not available to the public.

Surface Plasmon Resonance (SPR), Quartz Crystal Microbalance (QCM), cantilever and electrochemical detectors are the most widespread platforms used for immunosensors. The main advantages of these, when compared with immunoassays such as ELISA (Enzyme-linked immunosorbent assay), is the label free detection and the opportunity for measuring biochemical interactions in real time. Label free is advantageous as compared with current immunoassays, where labelled biomolecules are required for detection. Some of the main advantages of label free detection include the possibility to study interactions without any modification of the biomolecules of interest, avoiding impact on their bioreactivity. The closer similarity to the wild type of biomolecules makes possible the application of natural ligands and substrates and finally the possibility to develop cheaper and faster immunoassays (Comley, 2004). In

addition, with label free detection kinetic and affinity constants can easily be obtained (Haga *et al.*, 2008, Katsamba *et al.*, 2006; Regnault *et al.*, 1998).

A crucial step for the development of successful biosensors is the immobilisation of biosensing elements. The main aim of the current work was the development of new matrices to immobilise biomolecules on sensor surfaces and prove that the resulting biosensors would be good enough for detection of target analyte in real samples (e.g. serum). Not successful immobilisation of biosensing element on sensor can result attenuation of its bioreactivity and consequently can affect significantly the performance of the sensor. In immunosensors or enzymatic sensors, proteins are either physically adsorbed onto the sensor surface (Predki, 2004) or covalently attached via amino or thiol groups (Kusnezow and Hoheisel, 2003). Biosensing elements can be also attached on the sensor surface via DNA hybridisation (Ladd *et al.*, 2004), electrostatic interactions (Koubova *et al.*, 2001) or through high affinity interactions such as biotin-avidin (Busse *et al.*, 2002) and histidine-chelated metal ion between the tag and an immobilised capture molecule (Zhen *et al.*, 2006). Common immobilisation methods include direct attachment of receptors/ligands onto gold surfaces or through the application of an intermediate matrix such as polymers, hydrogels or self-assembled monolayer, to which the biomolecules are subsequently attached. In some cases attempts have been made to achieve oriented immobilisation, where the receptor is attached to a surface by a particular part of the molecule (Vikholm, 2005; Cretich *et al*., 2006). Particular care needs to be taken to protect the immobilised receptors/ligands from denaturing processes during or post immobilisation (Butler, 2000). For this reason, direct immobilisation of proteins or other biomolecules on metal surfaces is not recommended because it results in losing 90% of their bioreactivity (Schasfoort and Tudos, 2008). The applied immobilisation

needs to be fast, cheap and most importantly should not affect the biorecognition activity of the immobilised receptor. In order to achieve all these requirements, a novel protocol for the covalent immobilisation of biomolecules containing primary amines using both inexpensive SAMs (based on commercial polythiol monomers) and novel polymers was developed here. The receptors immobilisation is based on the reaction between primary amines, thiol and o-phthaldialdehyde (Simons and Jonson, 1978). The performance of the immobilisation protocols was assessed by evaluating the binding Antibody (Ab) – Antigen (Ag) and by comparing the performance of newly developed surfaces with commercially established ones. Since the present work was a part of the European project "Biognosis" which aimed at the development of an immunosensor for early prostate cancer detection, part of the evaluation of the newly developed materials was performed by detecting prostate specific antigen (PSA), which is a prostate cancer biomarker. Detection of microcystin–LR and *Salmonella Typhimurium* (ST) cells was also performed using the novel polymer and SAMs (Self-Assembled Monolayers) respectively. The experiments were performed employing Biacore 3000, which is one of the most developed and reliable surface plasmon resonance (SPR) devices. At the end, a PSA detection in serum solutions was performed in order to assess the potential of the system for applications in clinical diagnosis.

CHAPTER 2

LITERATURE REVIEW

2.1. Biosensors

2.1.1. Introduction

Biosensors are of great interest for academic and corporate research groups from around the world and markets have grown dramatically in recent years (Vikholm *et al.*, 2005; Sadana, 2006). There is prediction that biosensors market worldwide will further expand and it will reach $6.1 bilion by 2012. According to IUPAC (International Union of Pure and Applied Chemistry) biosensor can be defined as a "device that uses specific biochemical reactions mediated by isolated enzymes, immunosystems, tissues, organelles or whole cells to detect chemical compounds usually by electrical, thermal or optical signals". The difference of biosensor with a chemical sensor stands in the fact that it applies a biological recognition element, typically a protein, a peptide or an oligonucleotide. (Eggins, 2002). The great advantage of biosensors is the opportunity to measure target analytes without applying reagents, which assist the development of low cost, environmentally friendly and fast methods. The factors that need to be taken into consideration in order to assess the performance of a developed biosensor are (Eggins 2002):

(i) Selectivity: This characteristic is vital for a successful biosensor development and it is the ability to distinguish the analyte from other substances present in the sample, which can otherwise contribute to a signal resulting in false positive results. An example of this is the glucose biosensor where the application of ferrocenes as mediators allowed the reduction of the potential applied for the measurements, minimising the contribution of other blood components oxidation such as ascorbic acid in the recorded current (Eggins 1997). The operation of the transducer sometimes can also influence the selectivity.

(ii) Sensitivity range: The sensitivity should be at nanomolar level or sometimes even down to femtomolar level (10^{-15} M).

(iii) Accuracy. For reliable measurements and development of validated analytical method the coefficient of variation (CV) should be lower than ± 5%.

(iv) Nature of solution. Conditions like pH, temperature and ionic strength during sensor application should be taken into consideration.

(v) Response time. Biosensors usually show higher response times than other chemical sensors (usually < 30 s). For example the Glucose meter from Lifescan OneTouch® UltraSmart® has response time 5s (Lifescan).

(vi) Recovery time. This is the time which is required between analysis of different samples and it should be as short as possible (not more than 5-10 minutes).

(vii) The working lifetime is another important factor for a commercially successful biosensor since biosensing elements are usually expensive and the cost of biosensor analysis can be reduced only by extending their lifetime. Thus the application of stable materials and regeneration under mild conditions which enables the use of biosensor for several sample analysis is important.

The most well known example of a commercial biosensor is the direct measurement of glucose concentration in a blood sample. Roche Diagnostics, Lifescan, Abbott and Bayer dominate the market of glucose biosensors (Newman and Turner, 2005). The application of glucose meter is advantageous comparing with more 'common' methods, in which many preparation steps are necessary and each step may require reagent to treat the sample. The results are displayed usually in 5s and the volume of blood needed to perform the test is not more than 5 μl which causes minimal pain to the patient (Lifescan).

The earliest example of a “device” which could be considered as a biosensor is the canary in a cage, as used by miners to warn of poisonous gas such as carbon monoxide (US Mine Safety & Health Administration). Nowadays many biosensor applications have in common that they apply microorganisms, which can response to toxic compounds at very low concentration levels. This new technology can keep people aware about the safety of their environment. These biosensors can be applied for environmental monitoring, in water treatment facilities and clinical monitoring. The main advantages of biosensors in comparison to standard analytical methods such as chromatography, spectroscopy, electroanalytical techniques, are the speed of measurements and the simplicity of operation which should not require any specialised laboratory skills.

2.1.2. Biosensors Historical Review - Applications

The first requirement of biosensors raised in hospitals where the doctors needed a way to monitor patients in intensive care. The sphygmomanometer was one of the first sensors developed for blood pressure monitoring. The most important step for biosensors development was made by American scientists in 1962 (Clark and Lyons., 1962) as a result of work with oxygen electrodes by Clark (Clark, 1956) who is considered as the father of biosensors (Renneberg and Lisdat, 2007). In Clark’s work, it is described for first time how to make an electrochemical sensor after attachment of an enzyme onto transducers. Particularly glucose oxidase was attached on the oxygen electrode and the decrease of measured oxygen concentration was proportional to glucose concentration in the sample. The concentration of oxygen was calculated according to the resulted current (height of peak) at the applied potential which was around -0.7V. This invention was very important because it informed the

patient about the level of glucose in blood and this biosensor had a significant contribution on diabetic's life improvement. Based on Clark's pioneering work (Clark and Lyons, 1962) the first biosensor was commercialised in 1975 by the Yellow Springs Instrument Company (Ohio) and it was a glucose analyser based on the amperometric detection of hydrogen peroxide. In the last few years biosensors have quickly developed and have been increasingly employed in a wide range of applications where continuous measurements in biological media are required. The high market demand for such sensors has expanded, and has pushed forward the development of sensor technology in general (Sadana 2006).

In 1984, Cass *et al.* reported the use of ferrocene and its derivatives as an immobilised mediator for use with oxidoreductases in the construction of inexpensive enzyme electrodes, which form the basis of a glucose meter (Cass *et al.*, 1984). Ferrocene or Ferrocene derivatives are used instead of a double membrane structure and are replacing O_2 as the natural electron donor-acceptor in the reaction pathway, allowing electron transfer from Glucose Oxidase (GOD) to the electrode surface at approximately 300 mV *vs* Ag/AgCl (Chuang *et al.*, 1997) or 160 mV *vs* saturated calomel electrode (SCE) (Cass *et al.*, 1984). The use of ferrocene is advantageous, because results in a lowered polarising potentials, which minimises the risk of oxidation of other electrochemically active solutes in blood samples such as diluted oxygen, ascorbic acid and uric acid. Thus most of the commercially available electrochemically based glucose meters employ ferrocenes as mediators. The electrochemically based glucose meters of Lifescan (US) OneTouch® can be considered as one of the most commercially successful hand-held biosensors for home use (Newman and Turner, 2005).

Another novel invention in biosensor field was the urea sensor, which was created by immobilising urease on the transducer surface. This sensor was developed by Guilbault and Motalvo (1969). In 1975, Lubbers and Optiz designed a biosensor able to detect carbon dioxide and oxygen. This idea was also used to create fibre optic biosensors. These scientists had a significant contribution in establishing biosensors as a research field of great interest. They developed biosensors that were based on very different technology showing that biosensors can be used in many areas and have a multipurpose use.

There is a wide variety of biosensor applications in medicine, food, environment, industrial processes industries, security and defence. The following prerequisites can transform a biosensor into an outstanding analytical tool for industrial or clinical applications and substitute existing analytical methods (Eggins, 2002): a) the availability of a suitable biorecognition element, b) the use of disposable portable detection systems, c) rapid results with in the timescale of diagnostic test, d) economical mass - production and consequently inexpensive for the customer, e) self calibrating which minimises action by user.

Health care is one of the main areas for chemical sensors and biosensors applications. The main achievements in this area has been the production of “one shot” biosensors, which are capable to determine one or more analytes simultaneously like glucose (Cass *et al.*, 1984), urea (Guilbault and Montalvo, 1969) and creatinine (Radomska *et al.*, 2004). In addition to that, biosensors measuring blood electrolytes or gases and metabolites, have been developed. These biosensors have found a wide range of applications in hospitals and mostly in cases of intensive care. Plenty of innovative devices have been made for potential applications in medicine like DNA chip for detection of genetic disorders (Deng *et al.*,2004), immunosensors for infection

diseases or disease markers detection (Konig and Gratzel, 1993a,b,c,d,e, 1994, 1995) and ion channel *sensors* (Goryll *et al.*,2003), which work by minimising the action of cell receptors.

Biosensor's application in environmental monitoring did not have the same development rate as in health care. Although, some applications are reported where simple chemicals can be measured in high sensitivity and specificity by using low cost equipment. Such measurements in water are BOD (biochemical oxygen demand), salinity, acidity, nitrate, phosphate, calcium and fluoride determination. BOD sensors incorporate intact microorganisms such as *Clostridium butyricum* and *Trichosporon cutaneu* (Eggins, 1997). The test can be accomplished in twenty minutes, which is very fast in comparison with standard BOD test, which takes 5 days to be completed. BOD sensors give the opportunity to plant managers to be informed immediately, but still in many cases BOD should be done according to standard protocols for legislative requirements. A current interest is the development of biosensors to detect endocrine disruptors, which can be active at very low concentration levels (ng l^{-1}), because of big variety oestrogens and oestrogenic mimics.

Some substances require a continuous real time monitoring and others only an occasional random monitoring. In addition to pollution detection, environmental monitoring sensors can meet important applications in farming, gardening, veterinary science and mining.

A restricted number of practical applications of laboratory based biosensors have been applied in food analysis. A main reason for the restricted development of biosensors in food analysis is due to the conservatism of the industry and low profit margins (Turner, 2000). Sensors mainly have been used in fermentation processes. Real time monitoring involves measurements such as temperature, pH, carbon dioxide and

oxygen. Biosensors have been also developed to determine analytes like sugars and alcohols (Cass *et al.*, 1984), penicillin levels in milk (Thrust *et al.*, 1996) and possibly undesirable by-products. This monitoring can have a significant contribution in improvement of product quality of raw material.

Defence Industry has an enormous interest in biosensors able to detect chemical warfare agents, nerve oil gases and other toxic agents. The use of inhibition of the enzyme acetyl cholinesterase produced a great interest, because it relates directly to the action of nerve agents on human body. Enzyme electrodes and enzyme reactors have been used for a couple of years for toxic agents detection (Eggins, 1997; Kiyoyuki *et al.*, 1995; Iqbal *et al.*, 2000) but they tend to be replaced by physical techniques measurement devices due to their high demand for consumables and restricted analyte range.

2.1.3. Future Challenges

Biosensors have been successfully used in a diversity of applications. In food industry the reduction of analysis time for analyte's determination during quality control process has significant impact in increasing productivity.

As it is well known biosensors are inexpensive devices created by combination of biological recognition with modern electronics or optoelectronics. It is of great importance for the commercialisation of biosensor to improve miniaturisation and mass production and to increase the number of analytes packaged in a single device by a multi channel format (Turner, 2000).

The greatest advantages of biosensors in terms of analysis are the exquisite sensitivity (in range of fM) and selectivity of biological molecules. Due to the use of biological molecule, a big disadvantage is however the instability. The size and complexity of

proteins makes them sensitive to degradation, especially in case when they are isolated from the natural environment. Many efforts have been spent in order to stabilise proteins incorporated into bioelectronic devices. The existence of commercial devices proves the success of these attempts. Despite that, unfortunately many molecules are not stable enough for commercial applications (Turner, 2000). The design and production of semi synthetic or synthetic analogues is very promising to solve this problem. New receptor elements such as aptamers and peptides are being created for biosensors by applying advanced computational techniques with combination of molecular biology and combinatorial chemistry. The purpose of these new receptors is that they are stable small molecules, which will substitute the larger counterparts with maintenance of molecular recognition characteristics. These novel synthetic receptors would also be more compatible with the fabrication of high-density microelectronic sensor arrays. Moreover the substitution of expensive antibodies with low cost synthetic biosensing elements can result to low-cost biosensors and subsequently expanding their applications. Despite the efforts on this direction antibodies application for immunoassays is still widespread due to the low affinity and specificity of these new receptors for the target analyte (Homola, 2008).

2.1.4. Principles of Detection

Most sensors have been developed by using electrochemical transducers, because they combine the advantages of simplicity of construction and low cost. Piezoelectric biosensors and optical biosensors based on the phenomenon piezoelectricity and surface plasmon resonance (SPR) have also developed and met widespread applications the last two decades. SPR exploits the property of gold and similar materials. In fact a thin layer of gold on a high refractive index glass surface can

absorb laser light, producing electron waves (surface plasmons) on the gold surface. This takes place only at a specific angle and wavelength of incident light and is highly dependent on the surface of the gold, in the way that binding of a target analyte to a receptor on the gold surface produces a measurable signal.

In general transducers can be subdivided in the following 4 main categories:

a) Electrochemical transducers: Electrochemical biosensor has been defined as a "self-contained integrated system, which is capable of providing specific quantitative or semi quantitative information using a biological recognition element retained in direct spatial contact with an electric chemical transduction element" (Scheller, 2002). Electrochemical biosensors are mainly based on enzymatic catalysis of a reaction that produces ions. The sensor's design contains 2 electrodes, a reference electrode and a working electrode. In some applications the presence of counter electrode, which is usually made of inert material, allows connection to an electrolyte and application of a current to the working electrode. The reaction with target analyte takes place on the active electrode surface (working electrode), and the ions which are produced create a potential which is subtracted from that of the reference electrode to give the signal (Eggins, 1997). One example of a complete three electrodes electrochemical cell is a screen-printed electrode, which is usually used to develop biosensors. These screen printed electrodes are made of conducting polymers like PPy (Polypyrrole) (Malhodra *et al.*, 2006). The resulting biosensors, which were made by immobilising biomolecules on the active surface area of the screen printed electrode, exhibited desirable characteristics such as high sensitivity, robustness and accuracy.The detection limits achieved with these biosensors were down to nM level and they are comparable with HPLC and LC/MS methods with the advantage of having lack of requirements for rigorous sample preparation and expensive instrumentation.

Electrochemical immunosensors have been developed for clinical diagnostics, food safety and environmental monitoring. Recent examples of electrochemical immunosensors for food safety are: the detection of *Salmonella Typhimurium* and aflatoxin M1 in milk by Dr. Tothill's group at Cranfield University (Salam and Tothill, 2009; Parker and Tothill, 2009). The above immunosensors exhibited very low LODs, 20 cells ml^{-1} and 39 ng l^{-1} for *Salmonella Typhimurium* and aflatoxin M1 respectively. Electrochemical transducers can be subdivided in 4 categories which are:

i) Potentiometric: The measurement of cell potential occurs at zero current and it is achieved by the use of ion-selective electrodes. The logarithm of concentration of analyte which is being determined is proportional to the applied potential (Eggins, 2002)

ii) Voltammetric: In this case an increasing potential is applied till the oxidation of the analyte takes place and at this point a sharp rise of current gives a current peak. In case of knowing the appropriate potential in which the oxidation occurs, this value can be used directly and current is observed. This mode is called amperometry (Turner, 1989).

iii) Conductometric: The electrical conductivity of the solution normally is changing as a result of change in composition when the reaction occurs, and it can be measured electrically (Hall, 1990; Newman and Turner, 1994; Skinner and Hall, 1997).

iv) FET-based sensors: If the construction of above types of electrochemical transducers is developed on a silicon chip, field-effect transistor (FET) based sensors can be achieved. This method has mostly been used with potentiometric sensors, but could also be used with voltammetric or conductometric sensors (Eggins, 2002)

b) Piezoelectric or acoustic sensors are devices which utilise crystals which undergo a phase transformation when an electrical current is applied (Turner *et al.*, 1987). In fact when alternate current is applied to the crystal, this starts to vibrate at a characteristic frequency. This frequency of vibration is highly affected by physical and surface properties of the crystal. In gas phase there is a direct proportion between the mass of the material adsorbed or covalently attached on the surface of the crystal and its frequency.

c) SPR detections based on phenomenon of plasmon resonance. Plasmon resonance takes place at a particular wavelength (this is why laser light is used as source for excitation) and under Total Internal Reflection condition at a particular angle. The angle in which plasmon resonance phenomenon takes place depends on refractive index of the gold surface. In case of binding or interaction of biomolecules with the gold surface the refractive index and therefore the angle changes and this makes the measurement of biological interactions possible in a high degree of sensitivity (Matsumoto, 1996).

d) Thermal Sensors. The sensitivity of thermal sensors relies on the measurement of heat, which is absorbed or produced during chemical or biochemical processes by sensitive thermistors. A thermal sensor relates the amount of heat, which is absorbed or produced, with the amount of analyte. Thermal sensors have not yet found a wide range of applications (Turner *et al.*, 1987).

Later on in this chapter, a detailed description of SPR and piezoelectric sensors, which are the sensors used for the current project, is reported.

2.2. Surface Plasmon Resonance (SPR)

2.2.1. Introduction

Surface plasmon resonance was firstly applied for bioaffinity studies in 1983 by Liedberg and coworkers (Liedberg *et al*., 1983) and the first commercial instrument based on SPR was introduced by Biacore in 1990 (Lofas and Johnson, 1990). SPR is an optical technique and the detection is based on monitoring refractive index changes as a result of mass deposition in close vicinity of sensor surface (around 300 nm). Since the first application this technology has made huge progress in terms of instrumentation and development. In fact a diversity of applications have been published and many commercially available SPR devices have been developed. This great interest and development of SPR sensors is due to high sensitivity, selectivity, speed and reliability in analyses. The most important attraction in SPR based sensors, is the detection of small molecules in low detection limits with high specificity. This is a very significant advantage, because most of the target analytes of food, environment and biomedical interest have low molecular weight, under 1000 Da in many cases (Miura et. al., 1997; Gobi *et. al.,* 2004; Shankaran *et. al.,* 2006a,b; Yu *et. al.,* 2005; Daly *et. al.,* 2000; Kim *et. al.,* 2006b). This has opened new horizons in identification and quantification of small molecules, which have been limited to the traditional chromatographic and spectroscopic methods and they usually require extensive sample preparation, time consuming procedures and highly trained personnel. In addition they are not compatible with real time, *in situ* or on-site detection application. SPR based sensors are simple, fast, cheap and innovative and therefore they are very promising for developing new analytical methodologies, which will overcome the disadvantages of the traditional technologies. SPR is

considered one of the most attractive optical signal transducer and its great advantage is the measurement of biomolecular interactions in real time in a label free environment (Green *et al.,* 2000; Homola *et al.,* 2002; Karlsson, 2004; Mullett *et al.,* 2000). The sensing element can be immobilised onto the sensor surface and corresponding analyte is free in the solution and passes over the surface. The sensogram displays the association and dissociation, which is measured in arbitrary units by monitoring the interfacial refractive index changes associated with any affinity binding interaction.

The use of SPR has provided major contributions in many research areas such as biomaterial characterisation, kinetics of antibody – analyte interactions, leading to ligand fishing in drug discovery and detection of a wide variety of chemical and biological substances (Homola *et. al.,* 1999; Green *et al.,* 2000; Homola *et al.,* 2002; Karlsson, 2004; Mullett *et al.,* 2000; Englebienne *et al.,* 2003; Windzor, 2003; Rich and Myszka, 2005). SPR is used to study protein binding (Ahmad *et al.,* 2003; Komolov *et al.,* 2006; Pei *et al.,* 2001), association / dissociation kinetics (Nordin *et al.,* 2005; Wintgens and Amiel, 2005; Wegner *et al.,* 2004) and affinity constants (Babol *et al.,* 2005; Huber *et al.,* 2004; Matsumoto *et al.* 2005). This consequently leads to wider application areas, such as molecular engineering (Calender, 2006; Kim *et al.,* 2006a; Kanoh *et al.,* 2006), food analysis (Sternesjo *et al.,* 1995; Haasnoot *et al.,* 2001; Spadavecchia *et al.,* 2005), clinical diagnosis (Inamori *et al.,* 2005; Thaler *et al.,* 2005; Haes *et al.,* 2005), proteomics (Natsume *et al.,* 2002; Kim *et al.,* 2005), environmental monitoring (Dillon *et al.,* 2003; Forzani *et al.,* 2005; Sesay and Cullen, 2001), bacteriology (Mader *et al.,* 2004; Zhang *et al.,* 2006; Oh *et al.,* 2005), virology (Rich and Myszka, 2003; Pizzaro *et al.,* 2001; Boltovets *et al.,* 2004), cell biology (Quinn *et al.,* 2000; Hide *et al.,* 2002; Oli *et al.,* 2006), drug discovery (Cimitan *et al.,*

2005; Nakatani *et al.*, 2001; Reddy *et al.*, 2006), warfare detection (Naimushin *et al.*, 2005; Shankaran *et al.*, 2006a,b), etc.

2.2.2. SPR Theory

In this section a description of basic principles of Surface Plasmon Resonance, which will help the reader to understand both what SPR is and under which conditions plasmon resonance occurs, is reported.

"Surface Plasmon Resonance is a physical process that can occur when plane-polarized light hits a metal film under total internal reflection conditions " (Biacore Technology Handbook, 1998).

Total Internal Reflection (TIR) takes place when a light beam hits a half circular prism. When the light passes from a denser medium to a less dense one, it is bent towards the plane of interface. At this point all the incoming light is reflected within the circular prism and the incidence angle reaches a critical angle.

Surface plasmon is created when the prism is coated with a thin film of a noble metal on the reflection site. Gold is the most commonly used noble metal. This is because it gives an SPR signal at convenient combinations of reflectance angle and wavelength. Moreover gold is chemically inert to solutions and this property makes it compatible with a variety of applications. As it was demonstrated by Otto in 1968 when the energy of the photon electrical field is the appropriate one, the incident photos can be adsorbed by electrons (outer shell and conduction band electrons). After absorbing the photons, electrons are converted into surface plasmons (Otto, 1968a,b).

In addition to having the right quantity of energy, surface plasmon resonance requires also the right momentum to take place. Plasmons have a characteristic momentum which depends on factors like the nature of the conducting film and the properties of the medium. Momentum can be described as a vector with both magnitude and

direction component. The right momentum can be achieved by changing the incident angle of photons. Therefore, surface plasmon resonance requires the proper energy and angle of incident light to occur. The intensity of reflected light in correlation with incident angle is shown on Fig. 2.1. The figure shows that at incident angle H_1 a total internal reflection with maximum reflected light density occurs. However, when the incident angle is approaching the point where the momentum is the proper one to create surface plasmons, the reflected light intensity reaches a minimum leaving a gap on scheme. This angle is determined as resonance angle or SPR angle (Schasfoort and Tudos, 2008).

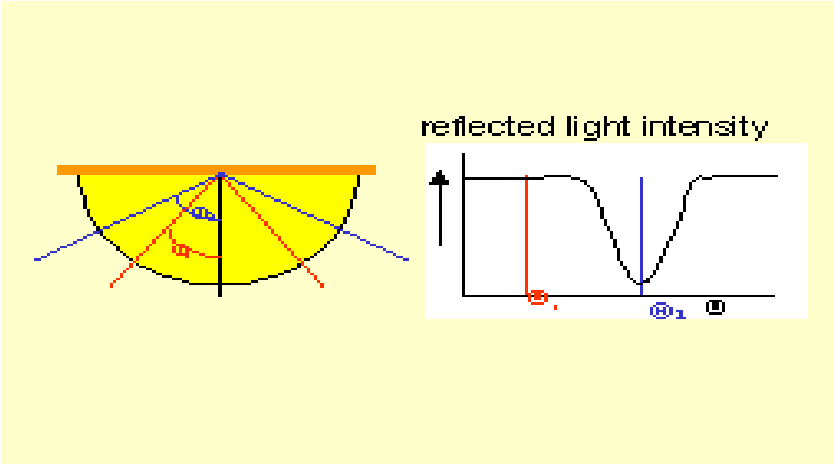


Fig. 2.1. *The dependence of reflected light intensity according to light incident angle (*BIACORE Technology Handbook*).*

At TIR an electric field is created by photons on the opposite site of interface. In case of plasmons another electric field is created which is comparable with the one created by photons. This electrical field is called “Evanescent Wave” and it is extended into the medium on the other side of the film. The definition evanescent wave is related to the property that the amplitude of the wavelength decreases exponentially by increasing the distance from the interface surface and it is completely attenuated at approximate distance of one light wavelength. The useful range of depth of an evanescent wave for measurements is estimated with in 300 nm of the sensor surface.

The evanescent field has got the same wavelength as the incident light. Heat can affect the evanescent wave, because it dissipates its energy (Stenberg *et al.*, 1991; Liedberg *et al.*, 1993). The way of how electric fields travel through medium depends on the medium properties. Light, for instance, travels according to the refractive index of the medium. Related with this, the reason for light refraction is that photons have a different velocity in different media.

According to this hypothesis, when the composition of the medium changes, the refractive index is also changing. Consequently the velocity and momentum of plasmons also change. This changing in the momentum causes change of the angle in which resonance takes place (Schasfoolt and Tuldos, 2008).

Resonant angle or angular SPR relies on precise measurement of this change (Markey, 1999). This is the most common type of SPR. Except for angular SPR a second type of SPR exists. In this case the angle is kept fixed and the wavelength can be varied till resonance occurs (Quinn *et al*., 2000). This type of SPR is called resonant wavelength SPR and it has not met a widespread use.

SPR depends mainly on three factors, which define the surface plasmon angle (Biacore Sensor Surface Handbook, 2003). These factors are:

i) Properties of metal film,

ii) Wavelength of the incident light

iii) Refractive index of the media.

Temperature can also affect the refractive index, thus the measurements should be performed at defined temperatures. In some cases this dependency can also be exploited (Roos *et al.*, 1998).

Metals which can be used for SPR applications need to have conduction band electrons capable of resonating with the incoming light at proper wavelength.

According to this demand, appropriate metals for SPR application are silver, gold, copper, aluminium, sodium and indium. In addition metals suitable for SPR applications should be free of oxides, sulphides and they should not to react with other molecules on exposure to the atmosphere or liquid. Among the above metals, gold and indium satisfy all the requirements, but gold is the only one practically used due to the high cost of indium. Other metals cannot be used because sodium is too reactive, aluminium and copper too broad in their SPR response (Fig. 2.2), and silver is easily oxidised.

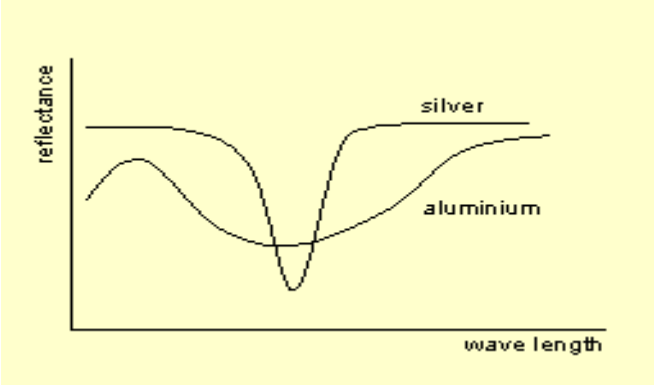


Fig. 2.2. *This scheme shows the broad aluminium SPR response in contrast with silver, which shows a satisfactory SPR response (*BIACORE Technology Handbook*).*

The main advantages of gold, which makes it the most appropriate metal for SPR applications, are the very big resistance to oxidation and other atmospheric contaminants, and the compatibility with plenty of modification systems.
Another important factor which should be considered is the thickness of gold which should be around 50 nm. According to the thickness of the gold layer the dip in refractive index becomes shallow or broader as it is shown in Fig. 2.3 (Naganda and Handa, 2000).

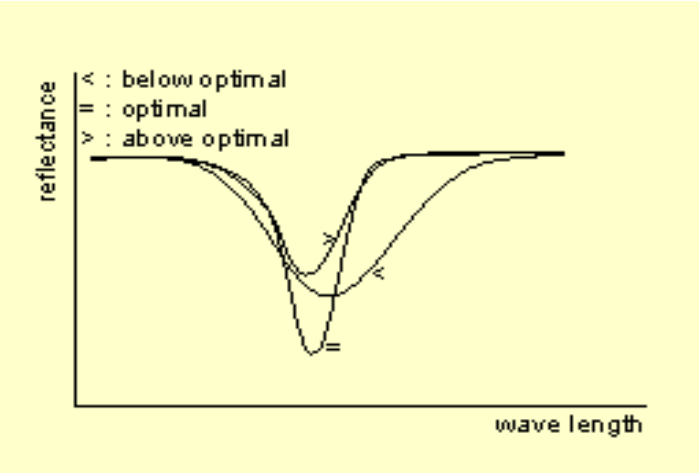


Fig. 2.3. *The effect of thickness of gold on the dip in refractive index. The optimal and most desirable are the sharpest and deepest one (BIACORE Technology Handbook).*

Light source should be monochromatic and p-polarized in order to obtain a sharp dip. In presence of not polarized light, this will increase the background intensity of reflected light and it will not give any contribution to the SPR (Naganda and Handa, 2000).

During experiments the metal film and incident light are kept constant and the SPR signal is directly dependent on the change of the refractive index of the medium on the sensor side of SPR surface. The refractive index of the medium is changing as a result of biomolecules binding. This is measured as change in a resonance angle or resonance wavelength. The correlation between the change in refractive index on the surface and the amount of molecules bound needs to be linear (Quinn *et al.*, 2000). This relation is referred as Refractive Index Increment (RII) and its value varies according to biomolecules interactions. For protein-protein interactions the refractive index increment (RII) is about 0.18-.019 (Davis and Wilson, 2000; Tumolo *et al.*, 2004). In some SPR machines the actual measured values (angle or wavelength) are converted into arbitrary ones, which are easy to display and interpret. For example

Biacore uses Resonance Units (RU), which is converted from the actual angle shift in reflected light.

In case of qualitative and comparative applications especially with small molecules it is important to be aware of RII. SPR response must be normalized for each compound for an accurate affinity ranking and correct stoichiometric measurements (Davis and Wilson, 2000). In simple protein – protein interactions, where the kinetic constants are determined, RII is not as significant for the generation of meaningful results.

2.2.3. SPR Instrumental Design

SPR instruments are classified in four major categories according the kind of plasmon resonance system which is used in each device. The appropriate SPR instrument can be chosen according to the application and analysis requirements. Below there is a brief description of each category:

i) Prism coupled sensors

There are three configurations of prism coupled system. The first configuration is Otto arrangement (Otto, 1968b). At this configuration there is a distance between the metal and the TIR surface. Between metal and TIR surface there is a lower refractive index medium. This configuration is usually applied for SPR studies in solid phase media.

The second configuration is Kretschmann (Kretschmann E. and Reather, H., 1968) and differs from the Otto configuration from the fact that the metal is directly on the top of TIR surface with out any space between them. This gives more efficient plasmon regeneration.

The third configuration is similar to the Otto arrangement but uses a special layer to enhance TIR. A resonant mirror is performing the coupling of the TIR light to

plasmons. A small layer of silica is deposited on a prism in which light is in TIR situation. A titanium layer is then deposited on the top of silica. The silica layer should be thin enough to allow generation of an evanescent field in the prism able to couple to the high refractive index titanium. This is called Frustrated Total Internal Reflection (FTIR) (Hall, 2001). The configuration reported in Fig. 2.4 shows the schematic description of the three types of prism coupled sensors.

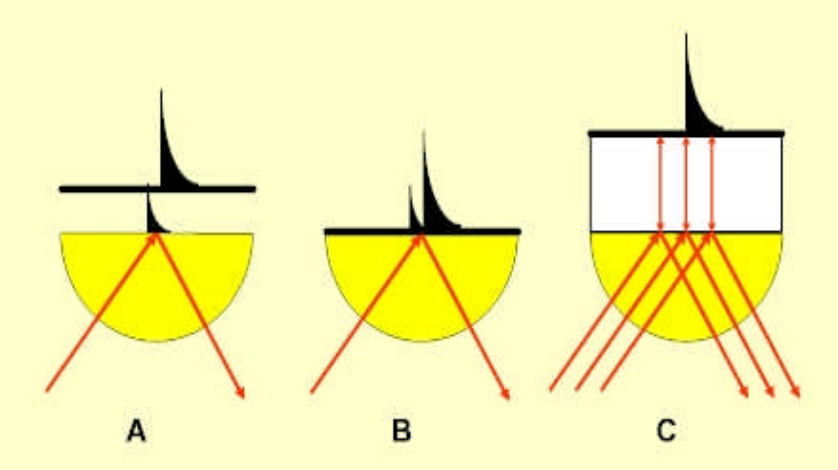


Fig. 2.4. *The three types of prism coupled sensors:* ***A)*** *Otto configuration* ***B)*** *Kretschmann configuration* ***C)*** *Frustrated Total Internal reflection configuration (www.sprpages.nl).*

ii) Fibre Optic Sensors

Fibre optic sensors consists of a multimode optical fibre and a surface plasmon metal such as silver (Jorgenson and Yee, 1993). The principle of detection stands in the fact that the light must enter the fibre at certain discrete angles in order to propagate through a multimode optical fibre. A different angle corresponds to a different mode or way of travel. Modes can be classified in low and high order ones. Higher order modes represent the entering of light at a steep angle, which bounces back and forth quickly. The energy is spreading mainly into the cladding. On the contrary, for lower

modes the light enters the fibre code at a shallow angle and consequently bounces back and forth quiet slowly. In case of fibre optic sensors, SPR sensing is achieved with a limited number of entry angles (Jorgenson and Yee, 1994).

Fibre optic sensors are advantageous comparing to the bulkier prism because of low cost. This allows also the application of fibre optic sensors for the development of cheap and disposable biosensors for medical or other sterile tasks. Another advantage is the small size of these sensors, which provide them higher flexibility for on the "field" applications. A fibre optic sensor is illustrated below in Fig. 2.5.

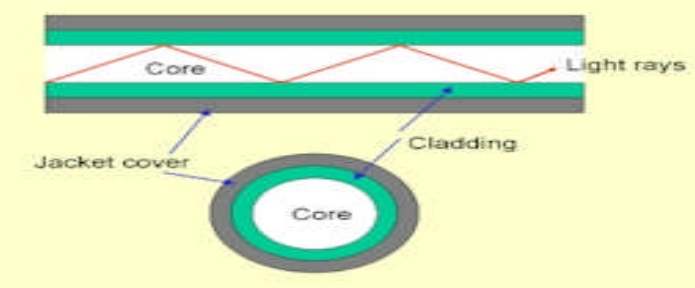


Fig. 2.5. *A schematic representation of a fibre optic sensor (www.sprpages.nl).*

iii) Grating Coupled Sensors

The use of grating coupled (GC) sensors was first published in 1983 (Lukosz and Tiefenthaler, 1983). The principle of GC systems operation is the so called diffraction grating (Hutley, 1982). The wavelength of resonance is determined by the period and the amplitude of grating. The grating substitutes the need for glass prism, which is necessary in traditional SPR. Gold is used for covering the grating. The surface of the chips illuminated by a light source and a CCD camera determines the reflected light intensity. A binding curve is created by changing the incident angle and measuring the reflected light intensity.

GC sensors existed in two configurations which are input and output grating couplers. In regard with input grating coupler, the presence of a light source is necessary to be shown though the substrate onto the GC. The coupling angle is affected by light and the detector which is situated at the end of the waveguide detects the presence of light. With respect to output grating coupler, a pre-coupled light source is used. When it reaches the GC the light is uncoupled at an angle dependent on n_{eff} (effective refractive index). The detector, which senses this reflection, is not attached to the GC.

iv) Optical Waveguide Sensors

The last category of SPR configuration is optical waveguide sensors. The function of these sensors occurs by varying the angle of incident light. A light wave is guided by the wave guide. When the light enters the regions with a grating and a thin metal overlay, it penetrates evanescently through the metal layer. The outcoming light is detected by photodiodes at the end of waveguide.(Lambeck, *et al.*,1992). The major advantages of optical waveguide sensors are the simplicity of controlling the optical bath, the small size and ruggedness

2.2.4. Advantages of SPR

SPR shows a plenty of advantages in comparison with other transduction techniques for a wide range of applications. The main advantages of SPR are mentioned below (Shankaran *et al.*. 2007):

1) The labelling of the reagents is not necessary for SPR applications. A fluorescent and the radioactive labelling are the most common ways to label a reagent. It is important to avoid labelling when it is possible. The reasons are that in many occasions labelling can be hazardous and also time consuming, especially during the

removal of excess reactants. If left, excess reactants can inhibit the analysis. The use of SPR makes possible to avoid this hazardous and time consuming methods. In addition to that, labelling proteins can affect their reactivity or specificity. These, consequently, reduces both qualitative (detectability, specificity, selectivity) and quantitative (kinetic and thermodynamic parameters, concentration analysis) information, which can be obtained from biological assays. Moreover, the fluorescent compounds are hydrophobic and this hydrophobicity increases the background binding, which can lead to positive error in measurements.

2) SPR gives a real time monitoring information about molecular interactions happening at the interface. This is very useful because it gives the opportunity of rapid evaluating analytical systems.

3) It is possible to achieve regeneration of the active sensor surface and subsequently a multiple use of the same sensor chip by injecting regeneration solutions, which should be able to remove the analyte without removing or deactivate the ligand. According to this requirement it is important to monitor carefully the regeneration process and in case of deactivation of the ligand if possible, it should be activated again.

4) By exploiting the advantage of specificity during biorecognition reactions, there is a real potential of developing an immunosensor, which will be capable to detect any analyte with negligible error measurement, avoiding complications in medical diagnosis.

5) The construction of prototype portable sensors was obtained thanks to the fact that SPR instrumentation gives the opportunity of miniaturisation and multispot detection. This makes possible the *in-situ* detection of clinical substances and on site detection of environmental contaminants.

6) The monitoring of small molecules (mainly with inhibition or competitive assay) with high sensitivity can be considered as the most significant application of SPR. This has a great impact in rising extensive use of biosensors in drug screening and security applications.

2.2.5. Immunoassay formats

Immunoassay techniques are classified in two main categories which are the heterogeneous and homogeneous techniques (Cooper, 2002; Luppa *et al.*, 2001; Marquette and Blum, 2006).

In heterogeneous techniques the antibody (Ab) is immobilised on a transducer and the reaction occurs on interface. On the other hand in homogeneous assays biochemical reactions take place in solution phase.

The heterogeneous format is the most commonly used for SPR immunoassays. The reason for that is the high selectivity of SPR phenomenon for surface bound reactions, which take place not more than 300 nm from transducer surface. This gives important advantages like the increasing of surface capacity, better sensor signal intensities and quick detection.

There is a further classification of immunoassays, according to the choice of detection methodology, which is dependent on the nature of target analyte, analytical sample, sensitivity of analytical instrument and application (Luppa *et al.*, 2001; Marquette and Blum, 2006; Barcelo *et al.*, 2001).

The most widespread measurement formats are:

i) Direct assay is the most applied format. The analyte in solution binds to the immobilised biosensing element. It is preferred when the binding of analyte in

concentrations of interest produces satisfactory response (Nakamura *et al.,* 2003; Sonezaki *et al.,* 2000).

ii) Sandwich assay is used when the response from direct assay is inadequate. The signal is enhanced with binding of second antibody on the surface with captured analyte (Severs *et al.,* 1993; Wei *et al.,* 2003).

iii) Displacement assay is usually applied for the detection of small analytes by displacing the captured conjugated analyte (analogue) on the surface (Chegel *et al.,* 1998; Charles *et al.,* 2004)

iv) Indirect detection format is used for the detection of small analytes. It can be applied as competitive or inhibition assay. Competitive assay occurs when the sensing surface is coated with an antibody interacting with the analyte or a conjugated analyte which is added to the sample. After the addition of the conjugated analogue in the sample the analyte and its conjugated analogue compete for a limited number of binding sites on the surface. The analyte concentration is determined by the binding response which is inversely proportional to its concentration. Inhibition detection format takes place when a fixed concentration of an antibody with affinity to analyte is mixed with a sample containing an unknown concentration of analyte. Afterwards, the mixture is injected in the flow cell of the SPR sensor and passed over a sensor surface to which analyte or its analogue is immobilised. Noncomplexed antibodies are measured due to the fact that they bind to the analyte molecules immobilised on the sensor surface. The binding response is irriversely proportional to the concentration of analyte. (Miura *et al.,* 1997; Gobi *et al.,* 2004; Shankaran *et al.,* 2006b).

2.2.6. Applications of SPR immunosensors

The numerous advantages of SPR immunosensor has established this technology as one of the most promising and widespread for biomedical, food related, and environmental applications. These desirable characteristics, which have had a great impact in the continuous growing and development of SPR immunosensor, are mainly the selectivity, rapid response, multianalyte detection, on-field analysis, versatility and flexibility. The different areas of SPR immunosensors applications are listed below:

i) Applications to medical diagnostics

The application of SPR immunosensor aims to allow direct and precise determination of analytes of biomedical interest, directly from biological samples by avoiding expensive and time consuming pre-treatment methods. By using SPR very small sample volumes are required for the analysis. The most relevant problem, which is faced in SPR applications in biology, is the fact that biological matrices contain high concentrations of a variety of proteins. These proteins can interfere with the sensor by producing a non specific binding or by preventing the specific biomolecules to interact with the analyte. This leads both to false positive and false negative results. It is important to face these problems during the immunosensor development in order to achieve a system where errors are negligible.

A wide range of analytes have been detected by using SPR immunosensors. This range includes cancer markers, antibodies against viral pathogens, drugs and drug induced antibodies, hormones, allergy markers, heart attack markers and other molecular biomarkers, DNA and RNA fragments and also live viruses or bacteria. Some of the biomedical applications reported in literature, with the detection limit of each method written in brackets, are for the following analytes: morphine (100 ppt)

(Miura *et al*, 1997; Sakai *et al.*, 1998); dopamine (85 ppt) (Kumbhat *et al.*, 2006); myoglobin (2.9 ppb) (Masson *et al.*, 2004); Staphylococcal Enterotoxin b (1 ppb) (Slavik *et al.*, 2002) ; Carbohydrate antigen (CA 19-9) (66.7 U ml^{-1}) (Chung *et al.*, 2006); Vascular Endothelial Growth Factor (VEGF) (1 pM) (Li et al., 2007); Interleukin - 8 (IL- 8) (0.02 ng ml^{-1} in buffer – 1.5 ng ml^{-1} in saliva samples) (Yang et al., 2005); Carcinoembryonic antigen (CEA) (0.5 ng ml^{-1}) (Tang *et al.*, 2006); anti-hepatitis B virus (h HBV) (0.64 nM) (Chung *et al.*, 2005); Oral anticoagulant warfarin (4 ng ml^{-1}) (Fitzpatrick and O' Kennedy, 2004); Insulin (Gobi *et al*., 2007); Human chorionic gonadotropin (HCG) (46 mIU ml^{-1}) (Chung *et al*., 2006); Troponin (cTn 1) (2.5 ng ml^{-1} direct assay - 0.25 ng ml^{-1} sandwich assay) (Wei *et al.*, 2003).

ii) Applications for food analysis

Nowadays in food industry there is a demand for continuous quality control in all stages of food production starting from raw materials, collection processing, storage, transportation till consuming.

Most of the food companies especially in developed countries have implemented the Hazard Analysis of Critical Control Points (HACCP) in order to keep high food quality. HACCP demands a continuous check during all stages of food production until the food is safely consumed as mentioned above. This has a relevant importance to ensure the maintenance of high quality of food. SPR sensor applications can substitute the current methods like HPLC, Mass Spectrometry or specific enzymatic methods, which are expensive, difficulty to use, with long time of analysis, that needs to be performed in the laboratory.

The analytes, which usually need to be defined for determining the quality, hygienic condition and purity of food products, include small molecular organic compounds,

fungal metabolites and either microbes or whole cell of microbes. Biosensors are very promising for developing inexpensive, accurate, user friendly, rapid and flexible methods for a wide range of applications related with food safety.

There are not as many examples of developed SPR immunosensors for analytes detection related to food quality as for medical diagnostics. Due to the need of quick and simple methods in food quality control during production process, Biacore developed and commercialised Biacore Q flex kits for the detection of several analytes food including vitamins (biotin, folic acid, vitamin $B_{2,}$ vitamin $B_{12,}$ pantothenic acid) and veterinary drug residues such as growth promoters (β- agonists) and antibiotics (sulphonamides, streptomycin, Chloramphenicol, tylosin) in animal products (Schasfoort and Tudos, 2008). The last few years has been a continuously increment of interest for developing immunoassays for detecting analytes related to food safety such as pathogens, toxins veterinary drugs, vitamins, hormones and chemical contaminants. Examples of developed immunoassays are reported below and they include mainly detection of pathogens, toxins, veterinary drugs, hormones, vitamins, proteins. The most remarkable examples of immunoassays developed for food safety are reported in Table 2.1.

Table 2.1. *SPR sensors developed for food safety.*

Analyte	***matrix***	***Detection limit***	***Reference***
Escherichia coli	buffer	10^3 cells ml^{-1}	Subramanian *et al.*, 2006
Salmonella enteritidis	buffer	10^6 cfu ml^{-1}	Koubova *et al.*, 2001
S. typhimurium	buffer	10^2 cells ml^{-1}	Oh *et al.*, 2004
S. typhimurium	milk	10^5 cells ml^{-1}	Mazumdar *et al.*, 2007
Listeria Monocytogenes	buffer	10^5 cells ml^{-1}	Leonard *et al.*, 2004
Staphylococcal enterotoxin B	milk	1 ng ml^{-1}	Nedelkov *et al.*, 2003
Staphylococcal enterotoxin A	raw eggs	1 ng ml^{-1}	Medina *et al.*, 2006
Aflatoxin B1	buffer	3 ng ml^{-1}	Daly *et al.*, 2000
Domoic acid	buffer	0.5 ng ml^{-1}	Yu *et al.*, 2005
Benzylpenicillin/ ampicillin/amoxicillin	raw milk	> 2 ng ml^{-1}	Cacciatore *et al.*, 2004
Cloxacillin	raw milk	15 ng ml^{-1}	Cacciatore *et al.*, 2004
Cephalexin	raw milk	50 ng ml^{-1}	Cacciatore *et al.*, 2004
Cefoperazone	raw milk	25 ng ml^{-1}	Cacciatore *et al.*, 2004
Chloramphenicol	Honey,prawns milk	0.2 ng g^{-1}	Ashwin *et al.*, 2005
Tetracycline	Honey, milk	15 ng ml^{-1}(milk) 25 ng g^{-1} (honey)	Moeller *et al.*, 2007
Riboflavin	Dairy products	70 ng ml^{-1}	Caelen *et al.*, 2004
Vitamin B5	Food matrix	4.4 ng ml^{-1}	Haughey *et al.*, 2005
Progesterone	Milk	0.6 ng ml^{-1}	Gillis *et al.*, 2006
4-nonylphenol	Fish	10 ng ml^{-1}	Samsonova *et al.*, 2004
Deoxynivalenol	Buffer	2.5 ng ml^{-1}	Tudos *et al.*, 2003
PCBs (Polychlorinated Bisphenyls)	Food Samples	1 ng g^{-1}	Tsutsumi *et al.*, 2008

iii) Applications for environmental analysis

In the field of environmental analysis there is a great interest in developing SPR immunoassays, which would highly advantageous in comparison to the conventional analytical methods. Some studies for the detection of dioxins, triazines, herbicides,

explosives and pesticides have already been reported. The use of SPR indirect competitive immunoassays is very common in this area of research. Moreover the interest about detecting environmental contaminants such as atrazine, dichloro diphenyl trichloroethane (DDT), 2,4-D,-Benzo (a) pyrene (BaP), biphenyl derivatives, 2,3,7,8- tetrachlorodibenzo-p-dioxin (TCDD), Trinitrotoluene (TNT), chlorpyrifos, etc. is continuously growing.

Some applications describing SPR immunosensors for environmental contaminants detection have also been already reported. One of the first applications mentioned in the literature describes the development of a SPR immunosensor for BaP detection, which is a carcinogenic endocrine disrupting chemical and potential marker for environmental pollution. The limit of detection was 0.01 ppb (Miura *et al.,* 2003). Atrazine was also detected at 1 pg ml^{-1} by mRNA based immunoassay developed by Lim and coworkers (Lim *et al*., 2000). More recently an immunosensor for the simultaneous detection of BaP and HBP (hydroxybiphenyl) has been developed with detection limit remaining at the same low level (Gobi and Miura, 2004). Detection of analytes like 2-4 Dichlorophenol (Gobi *et al.,* 2005; Svitel *et al,* 2000; Soh *et al,* 2003), TCDD, polychlorobiphenyl (PCB), atrazine (Shimomura *et al.,* 2001), TNT (Larsson *et al.,* 2006) have shown also very low detection limits by using indirect competitive assays. All these applications prove that SPR immunosensors can replace successfully with great advantages, the majority of conventional analytical methods for environmental analysis in very near future.

2.2.7. Future trends of SPR technology

SPR is a widespread technique which has met continuously increasing number of applications mainly in the last decade especially to investigate quantitative and

qualitative aspects of a variety of biomolecular interactions. It is quite likely that SPR in the very near future will be employed in even more applications and it will be able to replace most of the time consuming, environmentally unfriendly, traditional analytical methods. In order to point how this could become possible we should go through the factors which are currently slowing down the development of SPR immunosensors and consequently their applications in hospitals, factories and chemical laboratories as ISO (International Standard Organisation) approved methods.

The most limiting factor for the SPR development in terms of research and applications is the high cost of SPR automated systems, consumables and biosensing elements. There is a high possibility that the cost of all these will be reduced significantly in the near future. The reason is that new companies are emerging offering high quality SPR instruments, producing competition to Biacore which has dominated the market since 1990 (Schasfoort and Tudos, 2008). This competition will be essential for the price reduction of instruments and consumables (chips, maintenance kits). The reduced cost of instrument and consumables will encourage further research and consequently development of novel SPR-based assays for commercial applications. Another issue related with the high cost of immunoassays is the price of biosensing elements. Possible replacement of expensive antibodies and enzymes with synthetic receptors such as peptides or Molecular Imprinted Polymers (MIPs) can be crucial for developing and commercialised SPR based sensors. Another factor which could reduce cost and time of analysis is the opportunity to monitor many biomolecular interactions on the same chip. Thus Biacore has developed the Flex chip, which allows to monitor up to 400 interactions in one experiment (Biacore Flex Chip – Product Information).

In addition to the high cost, another inherent drawback of SPR is the presence of non specific binding. This is a limitation actually linked to SPR main advantage, which is label free detection. Thus determination of analytes in real samples especially for POC applications is challenging by using SPR. The reason is that the complex nature of the matrix, which contains high amount of proteins, lipids and other substances, could have a significant contribution to the recorded signal during analyte determination leading to false positive results. The specificity of the interaction could be assessed by combining SPR with MS (Krone et al., 1997), which is crucial in order to indentify the bound biomolecules on the surface. A way to study the specificity of the interactions on SPR and consequently to judge the reliability of the results obtained is the application of SPR Imaging. By using SPR imaging it is possible to obtain a microscopic view of the sensor surface and additionally to define regions of interest in order to monitor many biomolecular interactions simultaneously (Jordan *et al.*, 1997).

More factors which could influence negatively the development of SPR based Point of Care Sensors are (Schasfoolt and Tudos, 2008):

i) For POC applications one of the advantages of SPR technology such as the determination of kinetic constants for biomoleculars interactions is not necessary;

ii) SPR instruments are bulky and they cannot be easily mass produced for high volume market;

iii) The cost of expensive labels is not an argument strong enough to substitute the current labelled tests with SPR – POC devices.

Considering the prospective of SPR, the possibility of combining SPR with other techniques in order to eliminate the drawbacks listed above and the continuously

increasing number of analytes detected by SPR, there is great potential for SPR to replace traditional analytical techniques on a routine basis.

2.3. Piezoelectric Crystal Sensors

2.3.1. Introduction –Theory

Piezoelectricity was discovered in 1880 by the Curie brothers (Curie J. and Curie P., 1880) as a phenomenon where electric dipoles (developing a potential difference) are generated in anisotropic natural crystals subjected to mechanical stress. Many types of crystals exhibit the piezoelectric effect, but the electrical, mechanical, and chemical properties of quartz make it the most common crystal type used in analytical applications. Typically α quartz is used for crystal fabrication. The quartz crystal is sandwiched between two electrodes, which are generally composed of gold or silver and are prepared by thermal evaporation onto the quartz surface. An alternating electric field is developed in the crystal by applying a potential difference between the electrodes. With this applied voltage the physical orientation of the crystal lattice is distorted, resulting in a mechanical oscillation of a standing shear wave across the bulk of the quartz disk at a characteristic vibrational frequency (e.g. the crystal's natural resonant frequency). Only the region between the electrodes is piezoelectric active. The frequency of the crystal depends on the physical properties of the crystal itself and for analytical sensing device the proportional relation between the resonant frequency and the overall mass of the crystal is particularly important (Bunde *et al.*, 1998). In 1959 Sauerbrey (Sauerbrey, 1959) reported an empirical equation (equation 2.1) to describe the mass sensitivity of gas phase deposition on a piezoelectric crystal:

$$\Delta F = \frac{-2F^2 \Delta m}{A\sqrt{\mu_q \rho_q}} = -C\Delta m \qquad (2.1)$$

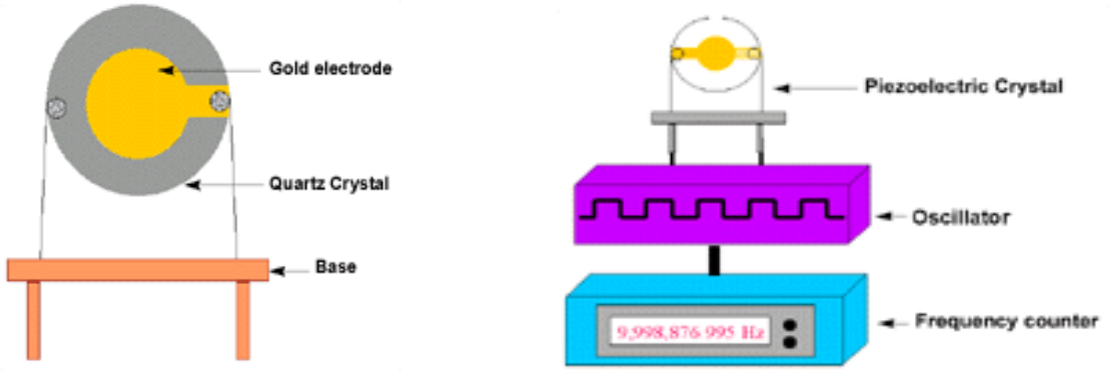
Equation 2.1. *Sauerbrey relation between mass and frequency of a quartz crystal in gas phase. ΔF is the measured frequency shift, in Hz; F is the fundamental resonant frequency (squared), in Hz; Δm is the mass change, in g; A is the piezoelectrically active area (area of electrode surface), in* cm^2*;* μ_q *is the shear modulus of quartz, in g* cm^{-2}*;* ρ_q *the density of the quartz, in g* cm^{-3} *and C is the mass sensitivity constant, in* $(sg)^{-1}$.

A change in the mass of the crystal per unit area results in a change in resonant frequency.

King has been the first person who reported in 1964 (King, 1964) the use of piezoelectric crystals as detectors of gasses such as benzene, toluene in gas chromatography. The next two decades have seen intensive efforts in using piezoelectric crystals to monitor a number of other gasses and vapours. In 1983 Guilbault (Guilbault, 1983) was the first to use a biological coating for direct assay in the gas phase.

The Sauerbrey's mass relation is not valid for a solution sensing system, since it is applicable only to gas phase mass deposition of rigid layers. Several investigators demonstrated clearly that the frequency response is affected by the viscosity and the density of the solution, even if the responses are not totally controlled by these factors. Later works reported that the frequency response can be affected by several factors such as conductivity and polarity of the solution, temperature, interfacial viscosity which is described in terms of hydrophilicity and hydrophobicity, crystal coating uniformity and the extent of crystal contact with the solution phase (Bunde *et al.*, 1998). Some researchers reported that the greatest sensitivity is in the centre of the

crystal and it is decreasing proportionally with increasing radius (Ward and Delawski, 1991). Hillier and Ward (1992) reported that mass changes occurring on the non-electrode portion should be avoided because they can not be calibrated due to inaccuracy of the sensitivity constant in this region. This problem could be solved by minimising and keeping constant the contact of the crystal's electrode portion with the solution and this can be easily achieved by using a flow cell (Bunde *et al.*, 1998). The two following schemes, 2.6a and 2.6b, show a quartz crystal in its holder and an integrated piezoelectric system respectively.



(2.6a) (2.6b)

Fig. 2.6. *Representative schemes of an integrated piezoelectric system with quartz crystal (2.6a) and the holder respectively (2.6b) (Kumar, 2000).*

For the application of piezoelectric crystals in affinity sensors the crystals are coated with a material that interacts selectively with a target substance. Two general experimental approaches have been used in most piezoelectric biosensing applications. One method, known as the 'dip and dry' technique (Guilbault et al., 1992, Yokoyama et al., 1995), consists in measuring the dry frequency of the crystal after the immobilisation of the biological component. Then, after the immersion in the reaction solution for a period of time sufficient for the binding reaction

(biocomponent-analyte), the crystal is removed from the solution, rinsed, dried and the frequency is recorded again. As the analyte interacts with the biocomponent, coated on crystal surface, the overall mass is changed producing a shift in frequency. The limitations of this approach are the tediousness of the dip and dry method and the lack of real-time data. The other general approach addresses these limitations as the analysis is conducted wholly in a solution phase. In this case a flow cell or some type of batch reaction cell is used to provide real-time data on the time course of binding events on the piezoelectric crystals (Barnes et al., 1991; Kurosawa et al., 1990; Minunni et al., 1994).

2.3.2. Applications of piezoelectric sensors

In the last two decades piezoelectric immunosensors have had a strong development and a wide range of applications due to the rising interest in studies of immunological reactions using quartz microbalances and various devices, based on quartz resonators. These immunosensors are able to determine mass changes due to the antigen binding on the immobilised antibody.

There is a significant amount of applications based on piezoelectric immunosensors, like detection of virus, bacteria, determination of proteins, nucleic acids assays, as well as affinity studies.

The opportunity of directly monitoring immunoreaction, with no need of labelling the analyte, can be considered the major advantage of piezoelectric immunosensors. The same advantage was mentioned before for SPR sensors. The non specific binding of the components of a reaction medium to the surface of a piezoelectric crystal is assigned as the main disadvantage of piezoelectric immunosensors. This causes low reliability and reproducibility of assays and this is an important factor, which should

be considered during the evaluation of the results obtained. In order to overcome this problem and eliminate the non specific binding, the unreacted groups of the receptor should be blocked with proteins like BSA (Bovine Serum Albumin) or amino contain compounds, like ethanolamine. In case the receptor does not cover the entire surface of the sensor, the spots of bare gold on the crystal, should be covered by thiol containing compounds. In addition the use of a control crystal could provide information about the non specific binding.

Piezoelectric immunosensors are suitable for detection of high molecular weight compounds. The possibility of direct determination of viruses, microorganisms can be considered a significant biosensor's advantage. Recently, many research groups have developed piezoelectric immunosensors for detection of a wide range of analytes for health care applications, bacteria, virus, toxins etc (Cooper and Singleton, 2007). Most of these methods are based on detection by immobilising antibodies on the modified gold surface. In the last few years applications of piezocrystal immunosensors based on DNA and RNA probes, as well as MIPs, which can substitute biomolecules have been reported.

Konig and Gratzel were two of the researchers who pioneered the development of piezoelectric immunosensors for direct determination of viruses, cells, bacteria, and their application in health care applications. Particularly, methods based on piezoelectric immunosensors for detection of human erythrocytes, T lymphocytes, granulocytes, rotavirus and bacteria, as well as hepatitis A, B viruses were reported by them (Konig and Gratzel, 1993a,b,c,d,e, 1994, 1995). In addition the same researchers described immunosensors for detection of virus and bacteria associated with acute diarrhoea (Konig and Gratzel, 1993c) with detection limit of 10^6 microorganisms per ml. Herpes simplex viruses type 1 was determined in serum by Cooper *et al.* (Cooper

et al., 2001) and the LOD was reported at 100 virions μl^{-1}. Varicella- zoster virus, Epstein – Barr virus and cytomegalovirus were determined with detection limit of 10^4 microorganisms per ml. Some more immunoassays for clinical applications involve detection of C-reactive protein (CRP) (Park *et al.*, 2003), fibrinogen (Aizawa *et al.*, 2003), bone morphogenic protein-2 (Michalzik *et al.*, 2005), cholinesterase inhibitors (Halamek *et al.*, 2005), a- fetoprotein (Tsai and Lin, 2005), ceruloplasmin (Wang *et al*., 2004) and various general sepsis markers (Carrigan *et al.*, 2005a,b).

Regarding food safety many immunoassays based on QCM sensors have been developed for detection of bacteria and toxins. Some of the most important include the detection and determination of *Salmonella enteritidis* (10^5 cells ml^{-1}) (Si *et al*., 2001), *Salmonella paratyphi* (170 CFU ml^{-1}) (Fung and Wong, 2001), *Salmonella typhimurium* (3.0×10^3 CFU ml^{-1}) (Oslen *et al*.., 2003), E. Coli (1.7×10^5 cells ml^{-1}) (Kim and Park, 2003), *pseudomonas aeruginosa* (10^5 cells ml^{-1}) (Bovenizer *et al.*, 1998), *Listeria Monocytogenes* (10^7 cells ml^{-1}) (Vaughan *et al*., 2001), cholera toxin (10^{-13} M) (Alfonta *et al.*, 2001) and polymyxin (2.5 pg ml^{-1}) (Yang and Chen, 2002)

In some other types of piezoelectric sensors the detection molecules were not antibodies, like in the majority of the applications, but were antigens, immune agents, nucleic acids and MIPs.

Examples of piezoimmunosensors, which employ immobilised antigens on modified surfaces include the sensors for anti-fluorescein antibody detection. In this case the antigen fluorescein was immobilised on the sensor surface by reaction of fluorescein isothiocyanate with cystamine layer. The detection limit for the corresponding antibody was 5 ng ml^{-1} (Cohen *et al*., 1996). Another example is the immobilisation of the antigen N-ε-2,4-dinitrophenyl-L-lysine (Blonder *et al.*, 1997) for detection of

corresponding antibodies. This enables the application of indirect assay for analyte determination in samples.

Some piezoelectric biosensors have been developed by employing DNA as sensing element. The opportunity to amplify the target DNA sequence by using polymerase chain reaction (PCR) before the measurement can decrease the detection limit of the method. Some research groups exploited the use of PCR for nucleic acids assays and they developed piezoelectric sensors for determination of genetotypes of human apolipoprotein E (Marrazza *et al*., 2001), *Escherichia coli* with low detection limit (only few bacterias per 100 ml) (Mo *et al*., 2002) and *Aeromonas hydrofila* (Tombelli *et al*., 2000).

In other piezoelectric sensors applications anti-ds DNA antibodies were used in order to amplify signal during detection of the Tay-Sachs disease (Bardea *et al.*, 1999). Other possible ways for achieving signal amplification and consequently higher sensitivity of method are: binding of biotinylated liposomes (Patolsky *et al*, 1999), deposition on gold nanoparticles (Weizmann *et al*., 2001) and also the intercalation of actinomycine D bound to magnetic nanoparticles (Zhang *et al*., 2002).

Piezoelectric DNA based sensors can be also applied for detection of genetically modified organisms (GMO) (Minunni *et al*., 2001). Despite the fact that piezoelectric sensors are best suited for high molecular weight compounds some applications for detection of small molecules are described. In case of small molecules as pesticides, drugs and hormones, when the direct assay exhibits low sensitivity to detect changes in frequency, the analyte is mixed with antibody for immunocomplex formation and the remaining binding sites of the antibody can interact subsequently with sensing surface, which is modified before with a derivative of the analyte.

In addition several other methods based on piezoelectric immunosensors were described, for detection of other small molecular weight analytes like: cocaine (100 pM) (Halamek *et al.*, 2005), NADP (220 μM) (Godber *et al.*, 2005), bile acids as taurodeoxycholate (LOD of 0.2 μmol l^{-1}) (Mo et al., 1999), fructose (LOD 0.5 mM) (Lau et al., 2000) and Indole acetic acid (5 nM) (Li *et al.*, 2002).

In last years the use of MIPs for simple determination of non polar analytes, with poor solubility in aqueous solutions, is rising. This is due to the fact that presence of organic solvents can deteriorate the affinity of antibody, whereas can improve performance of piezoelectric sensors based on MIPs, as the affinity between receptor (MIPs) and analyte is usually higher in organic solvent than in aqueous solutions (Horacek and Skadal, 2000). Thus molecularly imprinted polymers can be considered robust and stable biorecognition elements and this was shown with detection of terpenes (Percival *et al*., 2001). For the nucleotides adenosine 5'- monophosphate, cytosine 5'- monophosphate, guanosine 5'- monophosphate and uridine 5'- monophosphate molecular recognition sites were imprinted in an acrylamide - aminophenylboronic acid copolymer on the piezosensor surface and these provides a new way for sequencing of nucleic acids (Sallacan *et al*., 2002). Sensors based on MIPs were also developed for determination of paracetamol (Tan *et al*., 2001a), Dansylphenylalanine (Cao *et al.*, 2001), aminoantipyrine (Tan *et al*., 2001b), caffeine (Kobayashi et al., 2001), microcystin-LR (Chianella *et al.*, 2002), 4- Aminophenol (Karousos and Reddy, 2002), Acetaldehyde (Hirayama *et al.*, 2002), Hexachlorobenzene (Das *et al*., 2003) and L-glutamic acid (Feng *et al.*, 2004).

2.4. Modification of Surfaces and Immobilisation Methods

2.4.1 Introduction

The immobilisation of biomolecules on sensor surface can be considered a key step for a successful biosensor integrated system. A prerequisite necessary to obtain a successful immobilisation on a sensor surface is that the surface should be modified in a proper way for two basic reasons. The first reason is that biomolecules (proteins, DNA) should be immobilised in a way that maintains their bioactivity and specificity for biomolecular interactions. A direct immobilisation of proteins or other biomolecules on metal surfaces is for example not recommended as with this method only 10% of their bioreactivity is retained (Schasfoort and Tudos, 2008). The other reason is that a successful modification of surfaces results in an increase of selectivity by reduction of interferences, which are caused by non specific binding. A high non specific binding affects in a negative way the reproducibility and reliability of the method and also raises the detection limit.

Chemical modifications of sensor's surface are the most commonly used. Basically surface chemistry is applied in order to design the interface at the molecular level and consequently to control the interactions between the surface and the different species present in the analyte sample.

Chemical surface modifications can be classified mainly in two ways on the bases of the way species are attached to the sensor's surface. Modification techniques can be distinguished in covalent and non covalent. In case of the covalent approach, which is usually preferable, a chemical bond is formed between the surface and the attached species. This is regularly a non reversible process. The type of applied modification and the nature of the formed bond depend on the particular surface and the chemical

functional groups present on it. In non covalent modification strategies, the species are attached on the surface through Van der Waals interactions, which can be charge-charge, charge-dipole, dipole-dipole, induced dipole- dipole, induced dipole-induced dipole (Taylor and Schultz, 1996). These approaches are less specific than the covalent approaches. However they are easier to achieve compared to the covalent ones.

In the following sections different surface modification techniques and their main applications are described.

2.4.2. Covalent Modification of Surfaces

In covalent surface modification a chemical bond between the surface and some functional group takes place. The type of groups available for modification differs and depends on the material to be modified and the required pretreatments. The reaction between the organosilane and oxide or hydroxide groups on the surface is the most commonly applied reaction for covalent surface modification when the biochip is a glass substrate. Metal's oxidized surfaces like Al_2O_3, SiO_2, TiO_2, RuO_2 and SnO_2 are also an important class of surfaces suitable for modification with silanes. The reaction between the organosilane (R-Si-X) and the oxidized surface (M-OH) is successful under anhydrous conditions with the solution of the silane in appropriate solvent. One or more M-O-Si linkages per silane can occur and this depends on reactivity of silane and the applied conditions. The surface coverage of the silane is reported as

$1\text{-}5 \times 10^{-10}$ mol cm^{-2} and this is the reason for loosely packed surfaces with attached groups in a semifluid state (Suri and Mishra, 1996). Fig. 2.7 shows the chemistry of the described reaction.

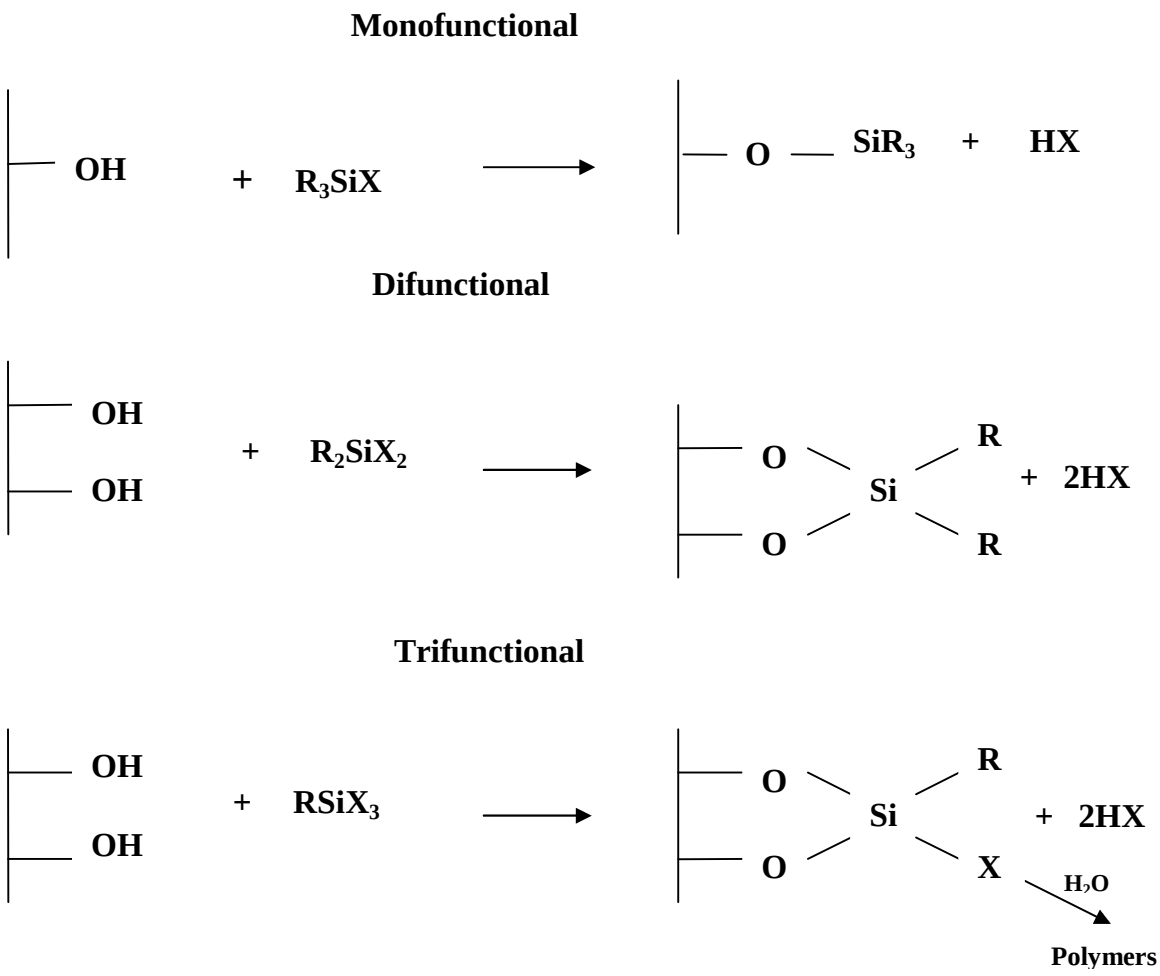


Figure 2.7. *The reaction between monofunctional, difunctional and trifunctional organosilanes.*

The coupling of organosilanes to the surface can be utilised in many ways due to the fact that polar oxide and hydroxide groups present on interface can be substituted by a variety of organic groups. This opportunity results in an alteration of interfacial properties of the surface. In addition to that silinisation changes the nature of the surface, making it hydrophobic or hydrophilic. Furthermore, silinisation can be used as a method for introducing other functional groups on to the surface to which covalent bonds can be formed and consequently other molecules can be attached. An example of this, is the use of alkylamine silanes in order to attach a wide variety of molecules like redox active groups, fluorescent groups, ion binding sites and other

types of groups which provide molecular functionality (Murray, 1984). Examples of chemistry which can be applied on alkylamine silanes are shown on Fig. 2.8.

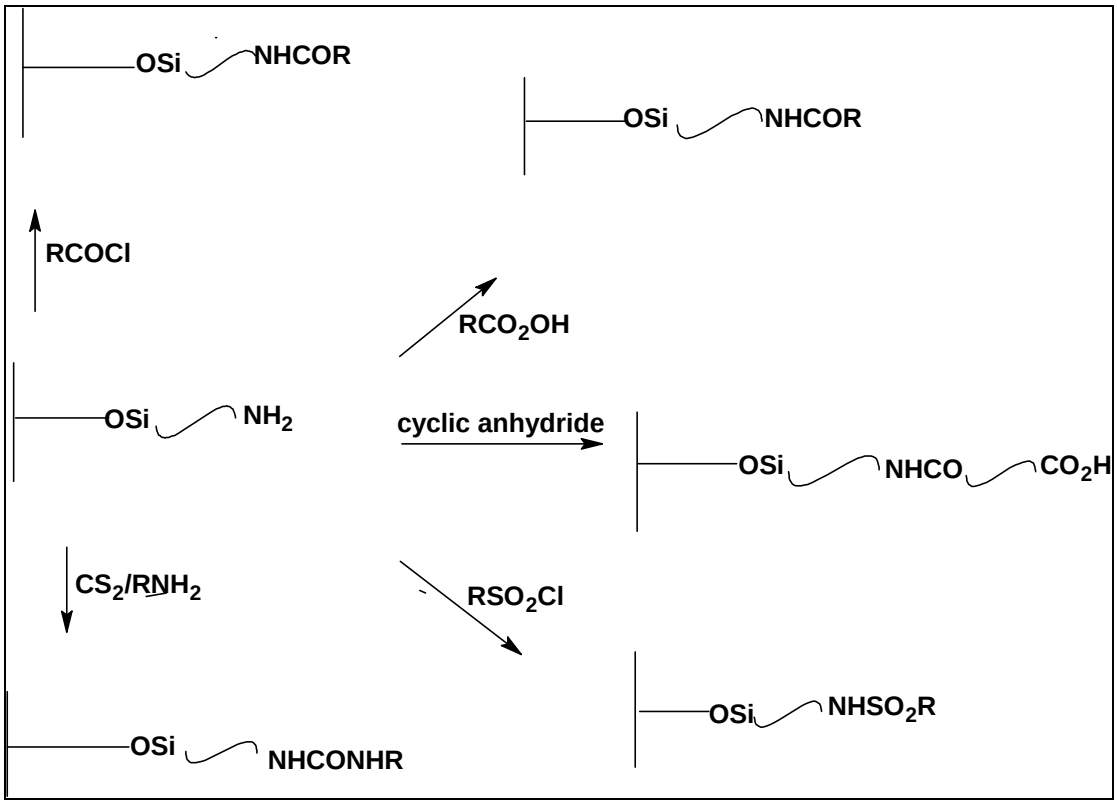


Fig. 2.8. *Examples of chemistry, which can be performed on alkylamine silane modified surfaces to introduce some further functional groups, providing flexibility in the development of novel immobilisation techniques.*

Polymeric silane films were also applied for covalently attachment to the surface, after cross – linking between the attached silanes, under particular conditions.

The modification of quartz crystal oscillators and acoustic devices surface is another important application of silinisation. The treatment of the surface by using aminopropyltriethoxysilane for obtaining higher sensitivity towards nitrobenzene derivatives has been reported (Heckl *et al.*, 1990). In this application initially the aminopropyltriethoxysilane was attached on the surface through its reaction with surface silanol groups on the quartz and the detection of the analyte (nitrobenzene derivatives) was through formation of hydrogen bonds with terminal amino

hydrogens. The same surface treatment has been applied as a pre-coating for biomolecules attachment to quartz crystal oscillators with modification through amine groups (Muramatsu *et al.,* 1986).

2.4.3. Self-Assembled monolayers and adsorption

Self assembled monolayer and adsorbed layer are less stable than covalent modification of surfaces due to the weaker and not bonding interactions between the surface and the adsorbing compounds. These approaches are mainly applied on metal surfaces and their main advantage stand on the fact that the adsorbed molecules often form close-packed arrays on the surface. On the other hand the disadvantage of these approaches is the stability of the surface modifications, as these are based on weak interactions and can easily peel off.

i) Chemisorption

Chemisorption on metal or carbon has been studied widely by many research groups and the adsorbed layers has been characterised in details. Examples of modified surfaces by chemisorption are platinum and carbon surfaces.

The surface of platinum can be modified by chemisorption of substituted alkenes. The interaction in this case is between π electrons of the alkene and metal surface. This process is irreversible. In case of carbon surface modification, the use of molecules with aromatic π- electrons which can interact with the π- electrons of the graphitic carbon is preferable. This modification was applied for deposition of redox mediators on carbon surfaces where catalysts were used for NADH oxidation in biosensors (Persson, 1990).

Armstrong *et al.* (1988) used chemisorption for modification of electrode surfaces in order to control and promote direct electrochemistry of large redox proteins like cytochrome c. Promoters are in general compounds consists of two main functional groups. The first one can be used as the provider of chemisorptive link to the metal surface and the other can create the covalent bond with the protein and consequently orient the protein at surface in a suitable way to maintain it bioactive. The major benefit of this surface modification is that the linkage between the two functional groups is rigid, which maintains the orientation of the binding groups towards the solution and consequently enabling the interaction with the protein.

Electrode surfaces for direct electrochemistry of redox proteins can be also modified by adsorption of amino acids and metal ions.

ii) Self Assembly

The modification of sensor chip during immunoassays development applying SPR or QCM detection format is based on the strong interaction between thiol gold and thiol groups, which is about 150 kj mol^{-1} (Dubois and Nuzzo, 1992). It was first applied by Nuzzo and Allara in 1983 and since the first application the technique has met widespread applications for gold surface modification (Nuzzo and Allara, 1983). Many studies of alkane thiols films on gold and platinum have taken place, due their ability to produce organised “self assembled” layers. This interaction leads to irreversible adsorption of monolayers, which form closely packed arrays. This happens as hydrocarbon tails of thiol compounds are oriented nearly perpendicular to the surface through hydrophobic interactions between adsorbed molecules (alkane tails) (Dubois and Nuzzo, 1992).

By exploiting the thiol adsorption on gold, several functional groups can be introduced onto gold surfaces in order to study biointeractions such as cell signalling

(Tidwell *et al*., 1997) and protein interactions (Holmin *et al*., 2001; Ostuni *et al.*, 2001).

A very important aspect which should be taken into consideration is the degree of perfection of the created film on the molecular scale in order to avoid pinholes and consequently non specific interactions during detection. The thiol SAM formation on gold requires only few minutes for most alkanethiols. Initially it is a disorder layer, which goes through changes for 1-2 days due to the adsorption and packing of more alkanethiols into the layer and molecules rearrangement to an optimal configuration. The required time for achieving the optimum ordered depends on different factors such as thiol concentration, the temperature, and the characteristics of applied alkanethiol (Schreiber, 2000). It is observed that by using thiols with longer chains a smaller number of pinholes on the film is obtained, because of stronger Van der Waals inter-chain interactions between the longer tails. According to this, the use of longer polymethylene chains were proposed in order to obtain better quality films with less pinholes. The use of electropolymerised films and other strategies were suggested for filling in these pinhole defects in the films (Finklea *et al.*, 1990).

The creation of self assembled monomolecular films and the control of their composition was exploited in many ways for controlling surface properties.

Song *et al* used alkanethiol for gold surface modification in order to achieve direct oxidation and reduction of cytochrome c which was strong adsorbed on alkanethiolate SAM in neutral phosphate buffer with low ionic strength (Song *et al.*, 1993). In a similar way ion sensitive monomolecular layers were formed by using immobilising ion binding sites within alkanethiol films (Rowe and Greager, 1991).

In another work it was demonstrated that it is possible to pattern self – assembled alkanethiol films, by applying photochemical techniques for partial modification of

the surface (Wollman *et al.*, 1993). In addition to this, it is also possible to control cell adhesion by using patterned alkanethiol.

Some other self assembled films, different from alkanethiols, are also reported. Some examples include long chain siloxane monolayers on glass, quartz, Al, Ge, Zn, Se (Sagiv, 1980; Maoz and Sagiv, 1984) pyridine derivatives on platinum (Stern *et al.*, 1989) and alkanoic acids on alumina (Allara and Nuzzo, 1985).

The direct immobilisation of macromolecules modified with alkanethiol, disulphide or sulphide anchors on gold and formation of SAM was successfully performed for compounds such as carbon nanotubes and fullerenes, porphyrins and phthalocyanines, carbohydrates, crown ethers and DNA. This immobilisation follows the same principle as straight chain alkanethiols. The main advantages of using macromolecules to create SAMs include the possibility to predict the distance between the functionalities, as this would be dependent on macromolecule size. Moreover there is the opportunity of introducing new functionalities on the modified surface. Furthermore by introducing more than one anchor on the surface it is possible to control the binding affinity. Lastly the space between the macromolecule and the surface can be modified by alternating the length of the linker (Beulen *et al.*, 1998; Schönherr *et al.*, 1999).

iii) Langmuir – Blodgett films

Langmuir- Blodgett technique is an alternative and efficient way to prepare ordered monolayer and multilayer films. The principle of this technique is that pinhole free monolayers are assembled at air – water interface and then transferred on to surface which needs to be modified. In order to achieve a successful, organised monolayer is important to balance the interactions between the molecules and air – water interface.

For Langmuir – Blodgett procedure, the molecules which create the films consist of two main groups the hydrophilic heads and the hydrophobic tails. The hydrophilic head groups are in contact with the water phase and hydrophobic tails stick out of the water.

A two dimensional close – packed molecular array is formed by using teflon or similar barriers, which can compress the spread molecules on water surface. Then this molecular array can be transferred to a solid support. An important prerequisite for a successful film preparation is the cleanliness during all stages of the procedure. The opportunity of controlling film thickness, orientation and composition at molecular level can be cited as the main advantages of Langmuir – Blodgett technique.

This technique was applied for developing films used as gating layers for ion detection and as films containing active gate molecules (Kuritara *et al.*, 1991). Also, an important application based on this technique is the fabrication of gas sensors by depositing layers of gas sensitive materials like metallopthalocyanines and substituted pthalocyanines at solid surfaces (Grate *et al.*, 1990)

iv) Phospholipid films

Phospholipid films are of great interest in research due to the fact that the resulting modified surfaces mimic the surfaces of biological membranes. This is an important advantage for biomedical applications because sensor's surfaces modified by phospholipids films are compatible with biological samples such as whole blood. This was proved by Chapman's work (1993) where hemocompatible surfaces were successfully created by modifying this surfaces with the same phospholipid head group, which exists on the outside of blood cells. Furthermore on the base of the same theory, Chapman and his group have used Langmuir – Blodgett technique and other

dip coating techniques to coat surfaces with mixtures of lecithin, diacetylene phospholipids and phosphorylchloride headgroup. In addition to these they prepared adsorbed coatings of phosphorylchlorine methacrylated based polymers on PVC, polyethylene cellulose and stainless steel (Chapman and Charles, 1992). They also applied phosphorylchlorine derivatives as plasticisers in polymers such as PVC and polyurethane (Hayward *et al.*, 1986).

Furthermore a variety of biomolecules were immobilised on surfaces coated with phospholipid monolayers. Some examples are the immobilisation of ionophores on mercury surfaces (Nelson, 1991) and immobilisation of glucose oxidase on biotinylated phospholipid layers (Snejdarkova *et al.*, 1993). Novel SPR applications involving the use of membranes and study their interaction with proteins can be found in the paper of Besenicar: "Surface plasmon resonance in protein-membrane interactions" (Besenicar, M. *et al.*, 2006). Biacore also commercialised the sensor chip L1 which enables the immobilisation of phospholipids on sensor surface (Biacore Sensor Surface Handbook) and further study of their interaction with proteins in biological samples.

v) Polymer coated surface

Another common method to modify surface is to coat them with polymer films or multilayer. The coating can occur even by covalent attachment to the underlying surface or by adsorption, which is actually the most common way of attaching polymers onto the surface. Adsorption can take place as a result of non covalent interactions between the large polymeric molecules and the surface through Van der Waals, polar interactions or ionic bonds.

The use of polymer films or multilayers is beneficial for surface coating because many monolayers with active sites can be formed and as a result larger active surfaces can be produced. This can contribute to an increasing of response and consequently, in higher sensitivity of the method, as well as extended lifetime. Another advantage of using polymer films is the formation of useful diffusional barriers to interferent species.

Three techniques are usually applied for coating surfaces with polymers. These techniques are spin coating, drop coating, dip coating and grafting. A brief description of each of these techniques is following (Taylor and Schultz, 1996).

Spin coating is the method in which a polymer solution is dropped onto the surface while this is spinning at high speed. Because of the spinning at high speed, the solution spreads out and forms a thin uniform film and after the evaporation of the solvent, the polymer film is remaining over the surface. The thickness of the film is defined by rotation speed and viscosity of the solution.

Another technique for surface coating with polymer, as it has been mentioned before, is the drop coating. A drop of polymer solution covers the surface and produces a thin polymer film after solvent evaporation. This way of coating is suitable only for small areas (up to 1 cm^2). A lack of homogeneity can occur and this is the main disadvantage of this method. In this case the thickness of the surface depends on polymer concentration in solution and droplet volume.

The last method, which was mentioned, is the dip coating. The aimed surface for modification is immersed in polymer solution and the polymer is absorbed on to the surface. In this case the thickness of the film can be controlled by time and polymer concentration in solution.

An alternative way to modify surfaces with polymeric films is the direct polymerisation of the film on to the surface or SAM which is polymer grafting (Schasfoot and Tudos, 2008). This is an easy and quick way to modify surfaces but at the same times it restricts any opportunity to purify and characterised the polymers, which are irreversible attached to the surfaces.

Polymers have met a variety of applications for modification of surfaces. They have been applied for chemical surface modification of sensors both for solution and gas phase measurements. Quartz crystal oscillators and SAW (Surface Acoustic Wave) devices were coated with a variety of polymers and polymer lipid mixtures (Hayashi *et al.*, 1990). Electrode surfaces have also been coated by modified polymers and functional polymers in order to promote electrolysis or to act as permselective barriers, for selectivity improvements.

These polymers can be classified in two main categories according to their functionality. The first type contains covalently bound redox active or other catalytic binding sites. The other ones include ion exchange polymers, such as Nafion and quaternised poly (vinylpyridine) and their functionality is based on the possibility that ions of opposite charges can be electrostatically entrapped.

Dendrimers have been applied on sensor surfaces to increase the active area of sensors. Dendrimers are branched structures and allow the introduction of a wide range of chemical functionalities which are constructed around the central core. In this way a 3-D structure is achieved with a significant increment of sensor surface. An example of dendrimers is Polyamidoamine (PAMAM) dendrimers developed and applied by Benters and colleagues (Benters *et al.*, 2001).

Another type of polymers, which are used widely for sensor's surface modification and lead to a 3-D structure and consequently high surface capacity, are hydrogels

(cross linked networks of polymers swollen with water). Their thickness can vary from 10-1000 nm. They are advantageous comparing to hydrophobic polymers due to the reduction of non specific binding and the fact that immobilised ligands are kept away from the surface and consequently this results in an increment of ligand accessibility and protection from deactivation (Schasfoort and Tudos, 2008).

Polycarboxylates, polyethers and polyols are mainly applied for surface grafted-hydrogels. The hydrogel density is a key factor to control its immobilisation capacity. High densities are usually preferable for analyte quantification due to the high amount of immobilised ligand which yields in high signals during analyte detection. On the other hand very high densities hydrogels can produce agglomerates in the upper layer of analytes over 10 kDa. This can inhibit the diffusion of further analytes into inner parts of the layer (Schasfoort and Tudos, 2008). The most commonly used hydrogel for sensor surface modifications is the carboxydextran, used also by Biacore on the commercially available SPR chip CM5 (Biacore Sensor Surface Handbook, 2003). Recently research groups have focused on MIPs grafting onto sensor surfaces which allows detection of analyte without any need of ligand immobilisation, as the polymer itself acts as receptor. An example of such application is the detection of domoic acid by using SPR which was demonstated by Lotierzo and coworkers. (Lotierzo *et al.*, 2004).

vi) Electrochemically generated films

Electrochemical deposition is a technique that exploits the change of polymers solubility after change in ionic charge of the polymer**.** Based on electrochemical deposition, polymeric films were formed and attached onto electrodes and sensor's surfaces by electrochemical polymerisation. During this procedure reactive radical

species are formed from monomers, in solution by oxidation or reduction at electrode surface. Afterwards these active species couple together to create the polymer film, which is attached on to electrode surfaces. The thickness of conducting polymer films can be controlled by polymerisation time. The major advantage of these polymers is the ability to attach different substituents to the monomers in order to tailor the polymer in an efficient way according to the application. Hence many research groups have studied and developed many sensors based on electrochemical polymerisation.

The nature and morphology of the films depends on many factors such as solvent, counter – ion and conditions of electrochemical polymerisation. The advantage of controlled polymerisation is that the entire process is electrochemically initiated and driven. The termination point can be adjusted by switching off the current.

The flexibility of controlling polymerisation was very attractive for many applications such as immobilisation of enzymes (Bartlett and Cooper, 1993). Another application was based on electro polymerisation deposition of heterostructures from solutions containing different monomers. This became possible with adjustment of deposition potential (Iyoda *et al.* 1991). Attachment of suitable substituents on electrochemically created polymers can increase their functionality. The attachment of crown ethers (Bartlett *et al.,* 1991) on polymer films, which have been prepared as ion selective material, are examples of enhancing functionality of electrochemically polymerised monomers.

After formation of films it is possible to modify them by covalent attachment of other compounds or by attentive over-oxidation to destroy the film conductivity. Electropolymerised conducting polymer films were also applied for gas sensitive chemoresistors. Some gas sensitive chemoresistors, which have been developed, were used for detection of ammonia, nitrogen oxides and organic vapours (Bartlett and

Ling-Chung, 1989). Another relevant application is the development of biosensor for monitoring odours and flavours (Slater *et al.,* 1993), (Pearce *et al.,* 1993). Moreover electropolymerised conducting polymers were also used for coating quartz crystal oscillators (Charlesworth *et al.*, 1993) and as gates of suspended gate field transistors (SGEFTs) (Josowicz and Janata, 1986). Polypyrrole (PPy) were applied and study for QCM and polyanile electropolymerised thin films for QCM and SPR by Suematsu *et al.* and Baba *et al.* respectively (Suematsu *et al.*, 2000; Baba *et al*., 2004).

2.4.4. Immobilisation methods

A variety of methods able to modify surfaces for obtaining active layers were described in previous sections (2.4.2, 2.4.3). The next step is of great importance and concerns the immobilisation of functional molecules (antibodies, DNA arrays) on the active layer for analyte detection. The immobilisation can be considered successful if the biological elements are oriented in a way in which their functionalities and bioactivity is ideal for a specific binding of the analyte. This is very important to achieve the highest possible sensitivity of method, better reproducibility and also lower cost.

Similarly to modification methods there are five major immobilisation methods which are applied for chemical sensors and biosensors. Particularly these methods are:

1) Covalent binding: Active component attaches the transducer by forming covalent bond. Examples of covalent immobilisation are the formation of peptide bond between carboxy or amino containing functional molecules with the appropriate modified surface, or linkage to activated surface groups (thiol, epoxy, amino, carboxylic etc.)

2) Entrapment: The active component is trapped physically into a film or coating.

3) Cross-linking: It is similar to entrapment with the addition of polymerisation agent (like glutaraldehyde) for providing additional chemical linkages between the active entrapped component and the film or coating.

4) Adsorption: The aimed functional group is immobilized on surface through hydrophobic, hydrophilic or ionic interactions.

5) Biological Binding: The active biomolecule attach the film or coating through a specific, biochemical interaction.

i) Covalent immobilisation

The most applicable method for attaching functional molecules on sensor's surface is by covalent bond formation between the transducer surface (or membrane or film coating the transducer) and functional molecule which can take place by using a variety of reactions. The most common way for covalent attachment of antibodies and other proteins is by peptide bond formation. This type of immobilisations is widely used for biosensors applications, because of higher resistance of sensor surface to changes of pH, ionic strength, and temperature. Moreover the immobilisation shows high stability in terms of reuse and recycling. On the other hand covalent immobilisation of biomolecules and especially of labile biomolecules, like enzymes or antibodies, can cause losses in the bioactivity.

The covalent attachment of receptor molecules on active sensor's surface can occur by utilising the presence of the appropriate functional groups in order to form the covalent bond. The most common used functional groups for this purpose include primary amines, thiol, hydroxyl and carboxylic acid groups. Proteins can be also immobilised on sensor's surfaces not only through the terminal groups, but also through the active groups found in arginine (guanidino), tyrosine (phenolic), histidine

(himidiazole), cysteine and cystine (sulphydryl), tryptofan (indole), and methionine (thioether) amino acids residues (Taylor and Schultz, 1996).

As it has been mentioned above peptide bond formation is the most frequent used immobilisation method for sensor preparation. Amino groups or other nucleophilic groups including thiol and phenolic groups, present on proteins or peptides, can form peptide bond by attacking electrophillic activated groups on the modified sensor surface. This reaction is also applied by Biacore for protein immobilisation on the carboxymethyldextran commercialised sensor chip CM5 (Lofas *and* Johnson, 1990) and it is illustrated in Fig. 2.9. The reaction requires activation of carboxylic groups of the surface with N- Ethyl- N'- (dimethylaminopropyl) carbodiimide (EDC)/ N-hydroxysuccinimide (NHS) in order to form the active succinimide esters. Afterwards biomolecules react spontaneously through their primary amino groups with reactive esters on the surface.

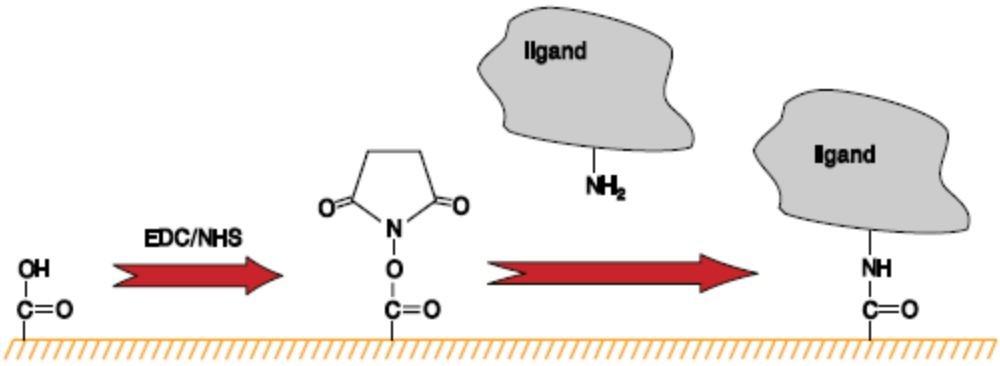


Fig. 2.9. *The amine coupling of ligands to the sensor surface is illustrated (Biacore Sensor Surface Handbook, 2003).*

Another reaction which was applied for amine coupling is the reaction of aldehydes with amino groups. Initially an unstable Schiff's base is formed, which can be further stabilised by reduction resulting in formation of a stable secondary amine linkage. Sodium borohydride and sodium cyanoborohydride are the most applied reagents for

this reaction, which can also occur efficiently at low and high pH. The main advantage of the reaction is based on the fact that can proceed in mild conditions and therefore is suitable for the immobilisation of sensitive ligands to polysaccharide surfaces (Massia and Stark, 2001). This reaction was firstly employed for sensor surfaces by MacBeath and Schreiber (MacBeath and Schreiber, 2000).

Aldehyde coupling is another important immobilisation method which is suitable for the immobilisation of glycoproteins and other glycoconjugates on sensor surfaces. The reaction, which is used for biomolecules immobilsation on Biacore carboxydextran sensors, is illustrated in Figure 2.10. The aldehyde coupling is recommended when the ligands contain sialic acid, as this group can be easily oxidated to an aldehyde by employing sodium periodate (O'Shannessy and Wilchek, 1990).

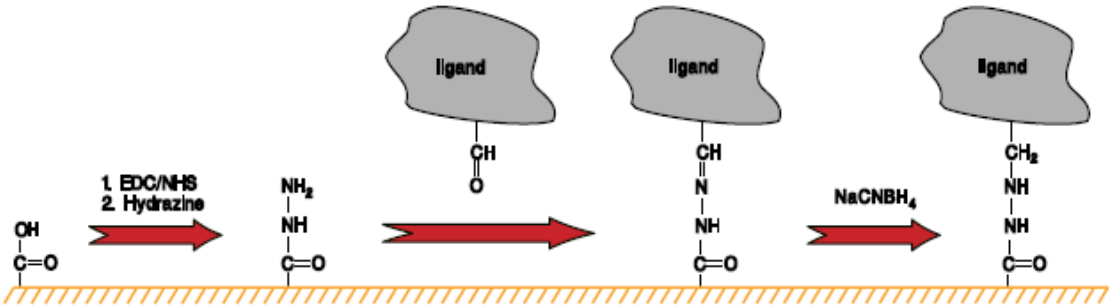


Fig. 2.10. *Aldehyde coupling of ligand on carboxydextran surface (Biacore Sensor Surface Handbook, 2003).*

Application of thiol chemistry is advantageous comparing to amine coupling because of the opportunity to achieve more oriented ligand immobilisation (Renberg *et al.*, 2005). It is also useful when the ligand is inactivated due to the presence of amino group on the paratope of the ligand. Thiol coupling exploits the reaction between thiol groups and active disulphide groups, which can be introduced on the ligand or on sensor surface. Finally a new mixed disulphide is formed and pyridyl disulphide is usually released as a leaving group during the reaction (Biacore Sensor Surface

Handbook, 2003). This reaction can take place in a wide range of pH (basic or acidic) or in low ionic strength buffer, which is important for the preconcentration of electroactive ligands. Immobilisation of ligands through thiol groups can take place also by maleimide coupling. This reaction is applied when the thiol disulphide exchange cannot be used because of the presence of reducing agents or high pH as the disulphide bond is not stable under these conditions. Maleimide coupling involves the reaction of thiol groups of the ligand with the maleimide reagents resulting in thiol coupling with the formation of thioethers. A neutral pH is usually applied to achieve high specificity of this coupling for sulphydryl groups since some cross reactivity with amino groups can occur at higher pH (Smyth *et al.*, 1964; Brewer and Riehm, 1966). The maleimide reagents which have been mainly employed and suggested by Biacore are N- [ε- maleimidocaproic acid]- hydrazide (EMCH) and N-[γ-maleimidobutyryloxy] sulpho- succinimide ester (sulpho-GMBS) (Biacore Sensor Surface Handbook, 2003). The procedure of maleimide coupling as it is applied on carboxydextran surfaces with the above maleimde reagents is illustrated in Fig. 2.11.

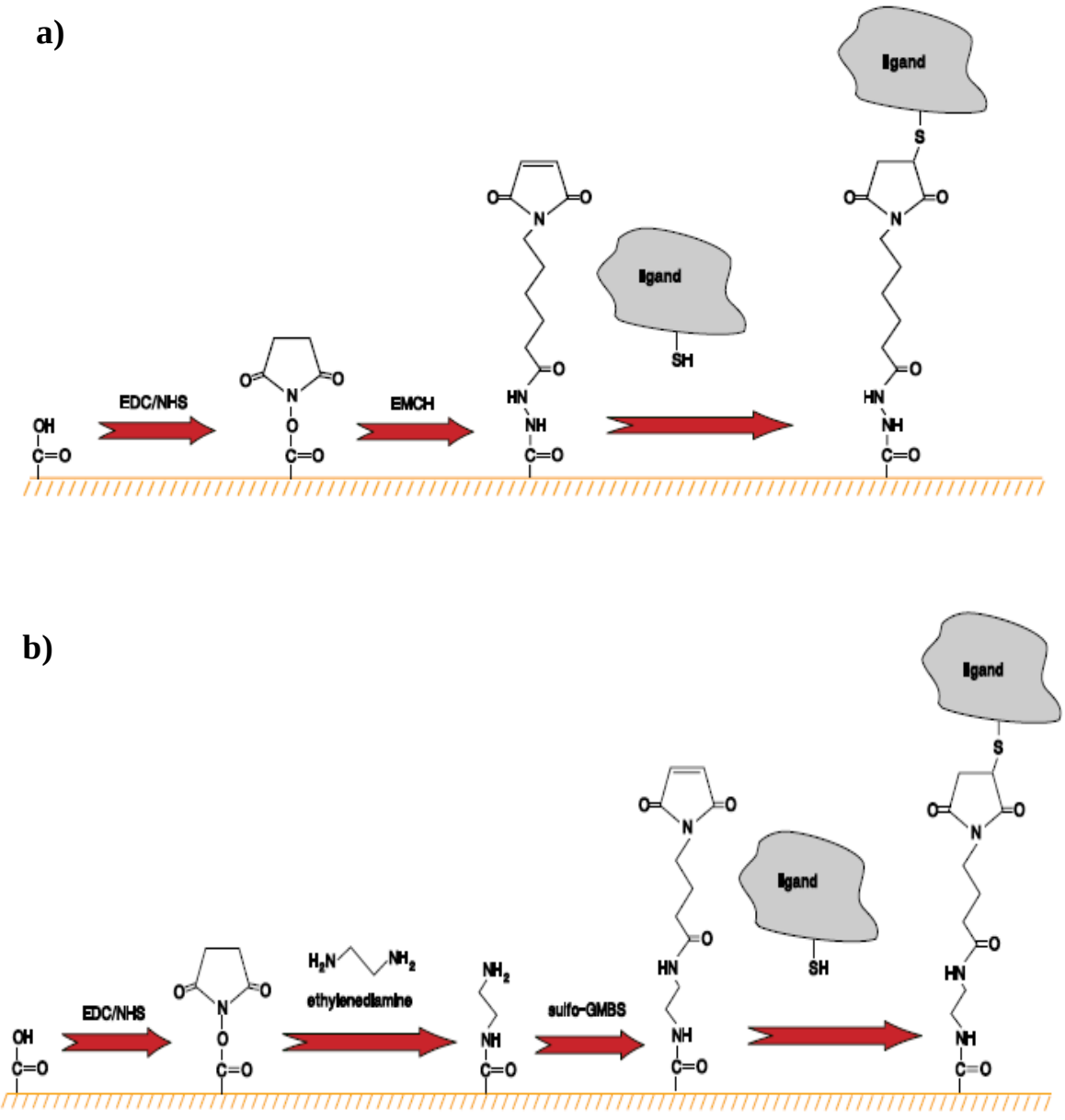


Figure 2.11. (a) *The maleimide coupling on carboxydextran sensor surface when EMCH is used as a maleimide reagent is illustrated* **(b)** *This figure exhibits the maleimide coupling on the same surface by using sulpho-GMBS as a maleimide reagent (Biacore Sensor Surface Handbook,2003).*

Epoxy-mediated immobilisation is another way of immobilising ligands on sensor surface and it was mainly applied for immobilising carbohydrate ligands in affinity

chromatography (Sundberg and Porath, 1974). This method can, however, be applied only if the ligand is reasonably stable in alkaline solutions and at temperatures of 70-90^{o}C since this is required according to the immobilisation protocol. In addition to the above methods the immobilisation of ligands on the surfaces can take place through radical substitution, photocoupling or other crosslinking chemistries (Schasfoort and Tudos, 2008).

Site - directed or directional immobilisation is used for biosensors applying antibodies as detection elements. Directional immobilisation is the immobilisation of biomolecules in a high – ordered and reproducible way. Hydrazine activation is a simple way to achieve directional immobilisation. In case of antibody – based biosensors, direct immobilisation is important because the immobilised antibodies should be oriented in a way that antigen binding sites are directed outward and easily accessible by antigen (analyte).

Such directional immobilisation can be completed after reaction of hydrazine activated transducer with antibody which is previously oxidized by applying sodium periodate (Domen *et al.*, 1990). The presence of sodium periodate aims to form the essential formyl groups by acting on vicinyl hydroxyl groups of sugars added in the heavy chains of the antibody (Ab). The antibody can be then immobilised by the following reaction:

$$-CONHNH_2 + OHC-Sugar-Ab \longrightarrow -CONH-N=C-Sugar-Ab + H_2O \quad (2.2)$$

An alternative way of antibody directional immobilisation is by reducing them with agents such as 2 – mercaptoethylamine (Hermason, *et al.*, 1992). This leads to the production of two half immunoglobulin molecules with free sulphydryl groups. After this modification, antibodies can be linked on the transducer activated surface by

covalent coupling using for example iodoacetyl activated surface as shown by the reaction below:

$$-NHCOCH_2CH_2-I + HS-Ab \longrightarrow NHCOCH_2CH_2-S-A + HI \qquad (2.3)$$

According to several studies on affinity chromatography and enzyme immobilisation, it has been observed that the distance between the immobilisation surface and the immobilised active molecule is essential for functionality (Cuatrecasas, 1970).This seem to be true also in case of sensors. The rest of surface modifications and immobilisation methods described below aim to maintain functionality of sensor's detection elements.

ii) Immobilisation by entrapment

Entrapment is a gentle method for biomolecules immobilisation. The fact that the reaction occurs under mild conditions and that non chemical treatment is required makes the method suitable for very labile molecules immobilisation, which may degrade or lose activity outside normal, physiological temperature or pH. Entrapment is not widely used due the weak bonding between the detector molecules and the matrix or transducer. The bond can be easily disrupted and this restricts any opportunity of regeneration.

Molecules to be immobilised by entrapment should be functionalised with the components of membrane or film prior to laying down the membrane or film on the sensor surface. It is also possible, that the molecules can be added after membrane or film attachment on the transducer.

Some examples of materials applied as films/ matrices for entrapment include starch, polyacrylamide, silicone rubber, polyvinyl alcohol and polyvinyl chloride.

The principal application involving entrapment immobilisation is the development of enzyme - electrode – biosensor (Guilbault and Kauffmann, 1987). An example of this kind of biosensor is the one for urea detection via ammonium ion electrode. The active molecule of this sensor is urease and it is immobilised by entrapment in an acrylamide film (Guilbault and Montalvo, 1969).

Most of the entrapment applications involve enzyme entrapment on hydrogels. An example of such application is the development of NADH and NADPH sensor based on entrapment of lipoamide dehydrogenase (LD) and glutathione reductase (GR) correspondingly, in a redox gel which was synthesised by the copolymerising vinylferrocene with acrylamide and *N,N*‘-methylenebisacrylamide (Bu *et al.*, 1998).

Highly labile biomolecules are also entrapped in hydrophobic bilayer lipid membranes and liposomes. These types of films and layers did not however show enough stability to be used for commercial biosensors and their application was restricted only at research level.

iii) Immobilisation by cross-linking

Cross linking immobilisation is a combination of covalent bonding and entrapment. Glutaraldehyde, hexamethylene, diisocyanate, difluorodinitrobenzene, bis-maleimidohexane, disuccinylsuberate and dimethyl suberimidate are the main cross linking agents. These agents have a double role, to polymerise a base layer or film and to attach the entrapped detection molecule in the layer of the film by formation of intermolecular bonds between the membrane and detection element. Usually detection biomolecules are quite stable when immobilised on layers formed by this method.

Cross linking was used for very labile receptor molecules immobilisation. Substantially, opiate and acetylcholine receptors were immobilised in a bovine serum albumin base containing phospholipids, detergent, antioxidants and cholesteryl ester using 2-5% glutaraldehyde (Taylor *et al.*, 1993).

iv) Immobilisation by adsorption

Adsorption is a traditional method for immobilisation of active biomolecules on sensor surface. It occurs when the solution of the molecule to be immobilised is applied to a membrane or film on the sensor transducer for a defined time period. The nature of the film or membrane can be hydrophobic, hydrophilic or may contain ionic groups according to the molecule to be immobilised. For most cases the immobilisation through adsorption is sensitive to changes of pH, temperature and media ion content and the immobilised molecules can be easily disadsorbed.

Three dimensional porous material like gel-pads were mainly applied. An example of gel-pads is the application of polypropylene membranes (PP) modified with polyaniline (PANI) where the ligands were immobilised with high affinity through hydrophobic and electrostatic interactions. The surface showed high compatibility towards many different proteins (Piletsky *et al.*, 2003). Some more ordinary applied materials for adsorption are silica, cellulose, acetate membranes and polymer like PVC and polystyrene.

One of the most remarkable applications based on ligand adsorption is the development of the glucose electrode biosensor by Clark and Lyons (1962) who had used polyethylene membrane for glucose oxidase adsorption.

v) Immobilisation by using biological binding

The use of biological binding is another method for the immobilisation of active biomolecules on sensor's surfaces. Biological binding shows many similarities to adsorption but with advantages in terms of specificity, bond strength and flexibility for directional immobilisation.

The formation of avidin – biotin complex is an important example of biological binding. It is one of strongest non covalent bonds with a very high affinity (Kd = 10^{15} M^{-1}), which allows the bond to withstand harsh conditions necessary for many biochemical assays (Craft *et al.*, 1998; Panayotou *et al.*, 1998). By employing this immobilisation method avidin or streptavidin is immobilised on sensor surface. As a result of it a high amount of binding sites available on the surface for attachment of biotin-conjugated ligands is created. This leads to a strong specific binding interaction and allows oriented coupling. On the other hand biotinilation of the ligand can alter its bioreactivity and a special consideration should be taken when it is employed for kinetic studies (Schasfoort and Tudos, 2008). An example of enzyme immobilisation achieved using biotin was described by Pantano and Kuhr (1993). Firstly the carboxylic acid surface was activated with the use of EDC in order to bind biotin through the diamine chain. Afterwards avidin was coupled to biotin immobilised on the surface, followed by a further coupling of biotylinated glutamate dehydrogenase (Fig. 2.12). This application exploited the very strong interaction between one avidin molecule and up to four biotin molecules. The formation of this complex is illustrated in Fig. 2.12.

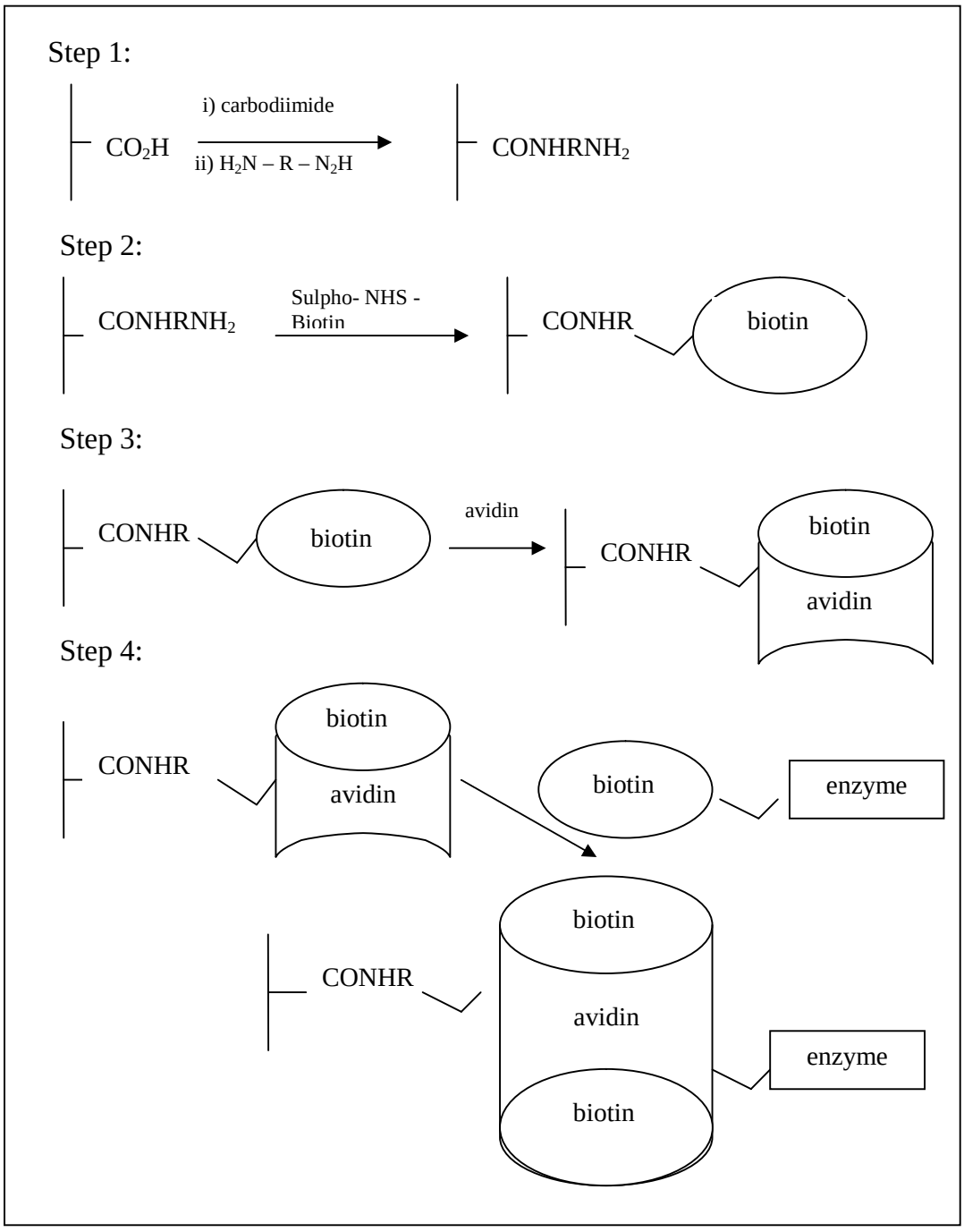


Fig. 2.12. *The reaction of attached biotin with avidin to form a biotin-avidin complex attached to the carbon surface. Afterwards the biotinylated enzyme is complexed to avidin to complete the immobilisation of modified enzyme to carbon fibre surface.*

Some other applications include the use of protein A or G, which binds specifically to the Fc region of antibodies. The use of protein A or G possesses the advantage of

providing a good access for the antigen onto the antibody paratope, since this is found on the Fab variable region (Quinn *et al.*, 1999,) far from the Fc region bound to the 'binding protein'.

DNA directed immobilisation is based on DNA chip technology. It is a relatively new method, where DNA hybridisation is utilised to achieve immobilisation of previously conjugated biomolecules with ss DNA. The affinity and the specificity of this reaction is high and it provides an elegant and flexible platform for SPR and other biochemical sensors. The main disadvantage is the requirement of conjugating the ligand with ss DNA (Ladd *et al.*, 2004).

Another common bioaffinity reaction, which has been applied for ligands immobilisation on sensor surfaces, is based on the reaction between histidine and chelated metal ions. Thus ligands are tagged with histidine (His) and afterwards they are able to chelate with Ni^{2+} ions in complex with nitrilotriacetic acid (NTA), which was previously immobilized on sensor surface (Zhen *et al.*, 2006). By using this approach it is possible to get highly ordered protein immobilisations due to the fact that the tag can be placed in defined positions of the protein. The low affinity of the reaction is the main drawback of the method, but at the same time allows reusability of the surface by introducing a competing ligand (imidazole) or a chelation compound (EDTA). The affinity of the reaction has also been improved by designing supramolecular multivalent chelator heads (MCH) with the presence of multiple NTA moieties and their binding with hexahistidine (H6) and decahistidine (H10). It has been shown that the increment of NTA moieties on the surface resulted in higher stability of the complex. Particularly the binding stability of chelator- oligohistidine complex was improved by 4 orders of magnitude comparing to the complex of mono His-NTA (Lata *et al.*, 2005).

Lastly another similar application involves the use of glutathione- S-transferase (GST) and it capture on anti-GST antibody surface (Biacore, Sensor Surface Handbook, 2003).

vi) Reaction applied for biomolecules immobilisation

The chemical reaction described in this section has been used for the immobilisation of biomolecules in this work. Reaction of o-phthaldehyde with thiols compounds in basic conditions results in the creation of a thioacetal. Further reaction of thioacetal with primary amines leads to a creation of a fluorescent isoindole (Simons and Johnson, 1978). This fluorescence reaction is commonly used for determination of amino acids.

This reaction is used in pre-column and post-column derivatisation in chromatography and it comprises the basis of procedures applied in commercial amino acid analysers. This reaction yields to a production of an isoindole derivative. It can be utilised for immobilisation of biomolecules through primary amino groups, which exist in amino acids such as lysine and arginine and the terminal groups of peptides. Immobilisation of DNA-RNA can also take place through the primary amino groups of DNA-RNA bases. The advantage of this immobilisation is that the compounds used for the formation of the thioacetal intermediate compounds can also contain groups that promote self-assembling of the polymer (or monomer) layer on a metal, preferable noble, transducer surface. In addition, once the thioacetal is formed it can bind amino-containing substances without additional activation. Further advantage of this method is the placement of appropriate amounts of ligands on detector surface using a very simple procedure. Another advantage is that maintenance of biorecognition activity of the ligands after immobilisation and minimisation of non-specific interactions

between the sample and the recognition element or other parts of the sensor can be easily achieved by choosing the right thiol monomer/polymer to form the thioacetal. The immobilisation conditions are also mild enough for biomolecules to maintain their bioreactivity. Fig. 2.13 shows the chemical reactions leading to the formation of the isoindole.

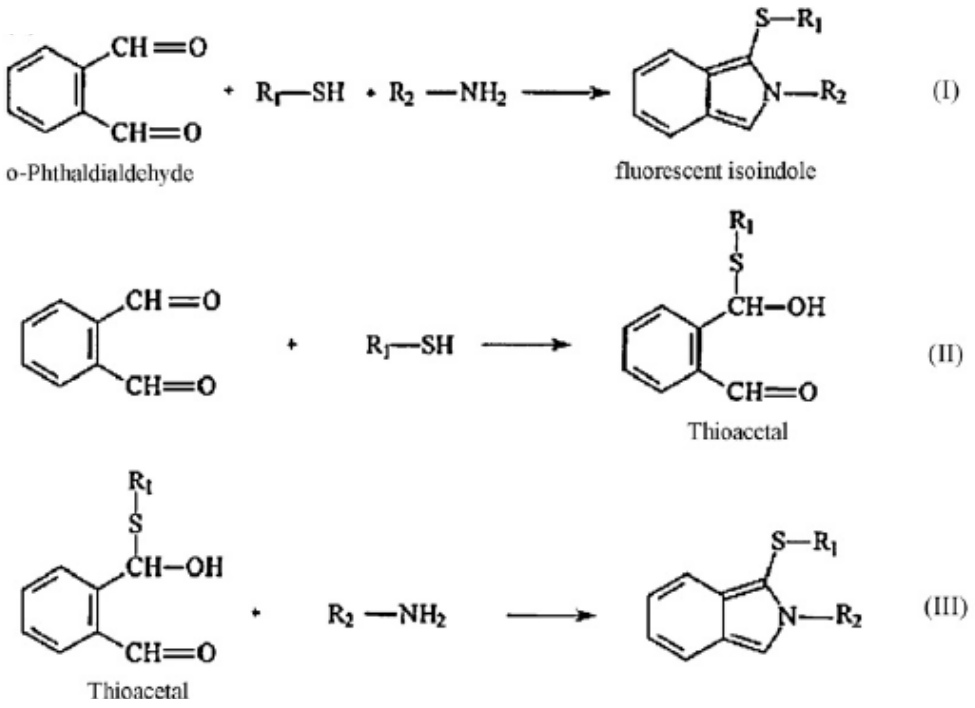


Fig. 2.13. *The chemistry steps involved in the biomolecules immobilisation. (I), hemithioacetal formation (II), formation of the fluorescent isoindole complex between hemithioacetal and primary amine (III) (Kyprianou et al., 2009).*

The optimum pH for this reaction is over 8. Basic pH is required to be near or over the isoelectric point of the thiol groups (negative charged thiol $–S^-$) in order to achieve the formation of thioacetal, which is an essential step of the reaction. A possible disadvantage of this method is the easy oxidation of the thiol groups. If this happens thiol groups are not available anymore for thioacetal formation and the reaction cannot take place. The formation of thioacetal groups prior immobilisation can be

considered a protection step to keep thiol groups active. Another way to keep thiol groups active is the removal of oxygen from solution and deactivation of metal ions (if present) by complex formation (EDTA), because metal ions act as a catalyst for thiol groups oxidation to disulfides.

2.5. Antibody - Antigen complex

The antibody – antigen biorecognition reaction was used in the majority of the work carried out in this thesis. This reaction is one of the most applied for biosensor development. A brief description of the antibody – antigen interaction is given below.

The antibody – antigen interaction occurs on two sites of the antibody, which are called paratopes. Each antibody can recognise a specific target, which is called antigen. The region of the antibody where the interaction takes place is called fragment antigen binding (Fab). It consists of one constant and one variable domain. Particularly the paratope is located at the amino terminal end of the antibody by the variable domains from the heavy and light chains (Putnam *et al.*, 1979). Paratopes can be considered similar to locks and they are specific for only one part of the antigen, called epitope, which can be thought as the key. This specific interaction is at the bases of the human's immune system, as makes an antibody able to tag a microbe or infected cell and protects organisms from inflammation and more serious diseases. For example if the antigen is a microbe, this can be deactivated by the specific antibody as this can block a part of the microbe that is essential for its survival and growth in the body.

Antibody- antigen binding is usually reversible and it consists mainly of non-covalent interactions such as hydrogen bonds, Van der Waals and electrostatic forces. The nature of interactions depends on the structure of the antibody, which varies with

isotype, and the structure of the antigen. The antibody can have either one (monovalent) binding interaction with an antigen, or multiple simultaneous (multivalent) interactions (Absolom and Van Oss, 1986).

Affinity of the antibody can be determined as the strength of the binding interaction between a single Fab region of an antibody and a single antigenic epitope.

In the present work antibodies anti-PSA (prostate specific antigen) were immobilised on functionalised sensors surfaces and the affinity of immobilised biomolecule tested.

2.6. Prostate Specific Antigen (PSA) – A cancer biomarker

Prostate specific antigen (PSA), which is the biormarker used extensively in this work for surface assesment, is called also as kallikrein III, seminin, semenogelase, γ-seminoprotein and P-30 antigen. It is a glycoprotein, which is produced almost exclusively by the prostate gland. The aim of PSA is to liquefy the semen and allows sperm to swim freely during ejaculation. It is also considered to be crucial in dissolving the cervical mucous cap and allow the sperm entrance. Biochemically it is a serine protease enzyme, and its gene is situated on the nineteenth chromosome (Lilja, 2003).

PSA is present in blood usually in a very low level, 0-4.0 ng ml^{-1}. High levels of PSA in blood can result from the presence of prostate cancer. A disadvantage of using PSA as a biomarker is linked to the fact that many other factors have also a great impact on PSA levels leading to a high percentage (30%) of false positive detections. Increment of PSA level can be a result of prostate infection, irritation, benign prostatic hypertrophy (enlargement) or hyperplasia or recent ejaculation. In some cases a prostate cancer was found even though the level of PSA was normal. In this case the detection was a false negative.

The majority of PSA amount in blood is bound to serum proteins and the small amount, which is not protein bound is called free PSA. In presence of prostate cancer the proportion of free PSA to total PSA is decreased. The possibility of cancer existence is increasing if the free to total amount of PSA is not more than 25%. Smaller ratio of free PSA to total PSA means higher possibility for prostate cancer. The combination of free and total PSA, detected in blood, can be considered a reliable diagnosis tool especially if its level is between 4 – 10 ng ml^{-1} (Catalona *et al.*, 1997). Annual screening test has been by U.S. Food and Drug Administration (FDA) in men older than 50. PSA levels between 4-10 ng ml^{-1} are considered suspicious and the possible presence of cancer is checked by a rectal ultrasound. As said above, though, arisen levels of PSA can be false positive-prone (7 out of 10 men in this category will still not have prostate cancer) and false negative-prone (2.5 out of 10 men with prostate cancer have no elevation in PSA) (Chuang *et al.*, 2007). The accuracy of the test is significantly improved by avoiding ejaculation 24 hours before the test. Routine screening is not recommended because it can be dangerous for health. The importance of regular prostate cancer test and the type of test recommended depends on genetic predisposition and consequently on racial, ethnic group and family history of prostate cancer. For example Africans have a higher risk of prostate cancer and at the same time Asian and Hispanic have a lower risk and this will influence the frequency of screening tests.

CHAPTER 3

POLYTHIOL MONOMERS

3.1. Introduction

In this chapter the development of Self Assembled Monolayers (SAMs) used for ligands immobilisation utilising a reaction between primary amines, thiol and o-phthaldialdehyde (OPA), see Fig. 2.13, (Simons and Jonson, 1978) is described. This reaction takes place without any pre-activation of the surface making it suitable for sensor/array fabrication. In this study several thiols molecules were tested using both a spectrofluorophotometer (recording fluorescence upon isoindole formation at the end of the reaction) and Surface Plasmon Resonance (SPR) with bovine serum albumin (BSA) as model protein. The following polythiol monomers were tested: trimethylolpropane tris (2-mercaptoacetate) (TMPTMA), pentaerythritol tetrakis (3-mercaptopropionate) (PETMP), 1,6-hexanedithiol (HDT), 1,9-nonanedithiol (NDT), 2,5-dimercapto-1,3,4-thiadiazole (DMTZ), DL-dithiothreitol (DTT) and their molecular structure is illustrated in Fig. 3.1. The above monomers were selected in order to assess how the different structures affect the performace after their immobilisation on sensor surfaces. PETMP and TMPTMA were applied in order to study the effect of amount of thiols on SAM formation. HDT and NDT were used due to the different length of alkane chain and to study its effect on resulting SAM. DTT, DMTZ were applied because they are water soluble and gives the opportunity of performing the attachment on the surface online on the Biacore. DTT, due to its structure, can produce hydrophilic SAMs, which usually result in low level of non specific adsorption and are consequently preferred for testing real samples. The effect of pH during SAM formation by employing the water soluble DTT monomer and during protein deposition on the surface was also studied. Among the thiols tested (Fig. 3.1), the ones demonstrating the most promising results (high surface capacity, reproducibility and low non specific binding), were applied for kinetic studies and

analyte detection. The compound, which contains 4 thiol groups (PETMP) showed satisfactory protein binding and was unaffected during surface regeneration. In addition the SAMs obtained with this molecule showed stability and negligible nonspecific binding when tested by SPR. The possibility to immobilise enzymes with acidic, neutral and basic isoelectric point on developed PETMP SAM was also studied.

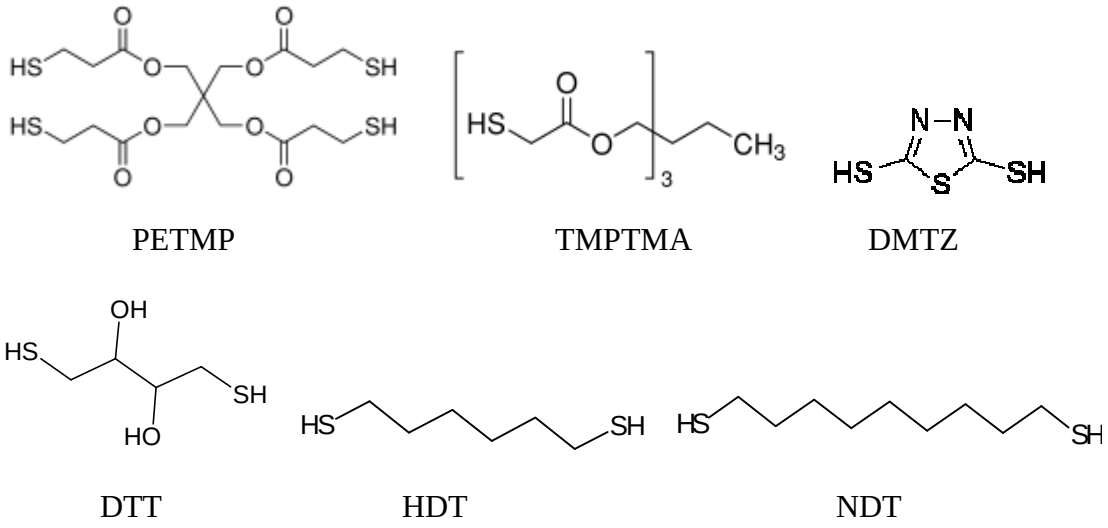


Fig. 3.1. *Structural representation of the polythiol monomers tested for SAM formations and fluorescent studies.*

As a final study the results were compared to those obtained using corresponding commercially available sensors (Biacore chip, C1). Our novel monolayer proved to possess equal and in some cases improved features compared to the commercially available chips. The immobilisation procedure on developed SAMs is depicted on Fig. 3.2.

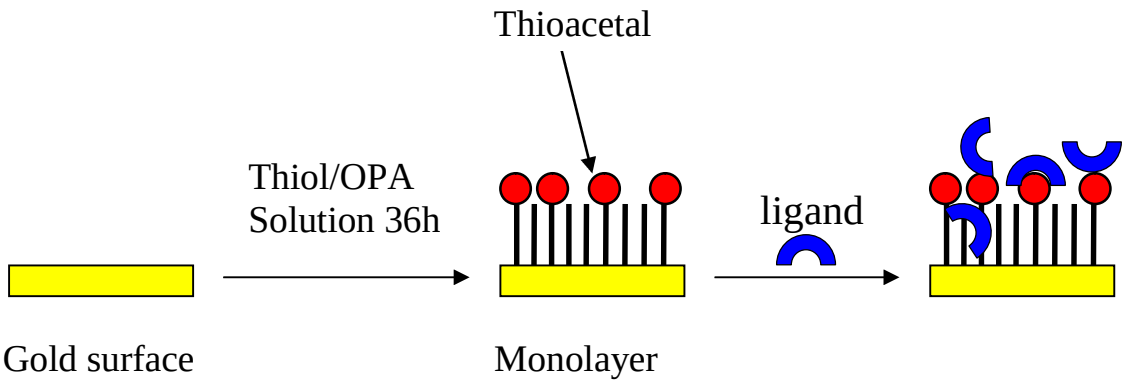


Fig. 3.2. *Schematic representation of biomolecules immobilisation on polythiol monolayer attached to the gold surface.*

The application of SAMs or multilayers (e.g. polymers) for the immobilisation of biomolecules has advantages and disadvantages and selection of one over the other depends on the application. Flat surfaces as SAMs are beneficial compared to polymeric layers (carboxydextran) in many cases; for instance when the analytes of interest are large molecules such as cells and viruses or for kinetic parameters determination, when a low amount of non specific binding is fundamental. For this latter a low level of immobilised ligand is recommended (Biacore Sensor Surface Handbook, 2003). The achievement of low or negligible nonspecific binding to sensor surface is a significant factor contributing to the success of sensor applications. In fact, nonspecific binding contribution during measurement leads to positive standard errors in analyte determination and causes errors in calculation of kinetic constants, especially for complex sample matrices like serum (Kusnezow and Hoheisel, 2003). In addition to immobilisation of a small amount of ligand, reduction of nonspecific binding can be also achieved by creating more hydrophilic sensor surfaces or by

including compounds such as polysaccharides/polyethylene glycol derivatives in the immobilisation steps (Masson *et al*., 2005). Another way for reducing nonspecific binding is the addition of surfactants, like P20, to analyte solutions (BIA applications Handbook).

3.2. Materials and Methods

3.2.1. Reagents

All compounds were obtained from commercial distributors and were of analytical or HPLC grade. Bovine serum albumin (lyophilised powder), IgG from bovine serum (95%), trimethylolpropane tris (2-mercaptoacetate) (TMPTMA), pentaerythritol tetrakis (3-mercaptopropionate) (PETMP), 1,6-hexanedithiol (HDT), 1,9-nonanedithiol (NDT), 2,5-dimercapto-1,3,4-thiadiazole (DMTZ), Triton X-100, the enzymes trypsin (lyophilised powder, from bovine pancreas), carbonic anhydrase (electrophoretically purified, dialysed and lyophilised), pepsin (lyophilised powder from porcine gastric mucosa) were purchased from Sigma–Aldrich (UK). o-Phthaldialdehyde (OPA) and D,L-dithiothreitol (DTT) were obtained by Fluka (UK). Mouse monoclonal antibody anti-PSA and anti *Salmonella typhimurium* (ST) as well as the native human prostate specific antigen (PSA) were purchased from AbD Serotec (UK). ST cells were kindly provided by Dr. Tothill research group (Cranfield Health, Cranfield University). Ethanolamine (ETA), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-hydroxy-succinimide (NHS), sodium dodecyl sulphate (SDS) solution (0.5%, v/v), P20 (10%, v/v), NaOH solution (0.2 M), 10 mM glycine–HCl, pH 2.5, SIA Kit Au and C1 chips were purchased from Biacore (Sweden). Solvents were supplied by Acros Organics (UK). The water was purified

by Milli-Q water system (Millipore, Bedford, MA, USA) and all the reagents used for Biacore experiments were filtered using a 0.22 μm teflon filter from Phenomenex®.

3.2.2. Assessment of thiol reactivity

An initial assessment of the reactivity of different thiols was performed by measuring the fluorescence produced by the isoindole derived from the reaction between the thioacetal (after reaction with OPA) and primary amine groups (Fig 3.2). Stock solutions were prepared by mixing thiols with OPA in molar ratio of thiol groups/OPA of 2:1 in DMF/ethanol (1:1). Although all stock solutions contained 1.0 mM of OPA the thiol molecules concentration varied in order to maintain the molar ratio thiol groups/OPA 2:1. The resulting fluorescence was recorded every 15 min, after 1:10 dilution of stock solutions in DMF/ethanol (1:1) and addition of 7.5 μl NH_4OH 6 M as a source of primary amino groups. The emission of the solutions was measured between 400 and 460 nm in a 3 cm^3 quartz cuvette using a RF-5301 PC spectrofluorophotometer (Shimadzu, Japan) with 360 nm as excitation wavelength. Maximum fluorescence signal was observed between 430 and 440 nm.

3.2.3. SPR testing

The performance of different thiols on SPR sensor surfaces was evaluated by using Biacore 3000 (Sweden). Biacore 3000 is a fully automated SPR instrument with four flow channels and capacity of 192 samples. The four channels can be serially connected and simultaneously monitored. It is one of the most reliable SPR instruments in the market and can provide reliable and high quality data (Schasfoort and Tudos, 2008). Thus most of reported SPR applications are based on Biacore

instruments. Au-coated chips (SIA Kit Au) purchased from Biacore (Sweden) were used for the experiments, which were performed at 25^{o}C. Biacore 3000 is illustrated in Fig. 3.3.

Fig. 3.3. *Biacore 3000 is illustrated in this figure (www.biacore.com)*

3.2.3.1. Treatment of gold chips–gold surface modification

Gold sensor chips, SIA Au (Biacore, Sweden) were used to assess the ability of polythiol/OPA monolayer to bind biomolecules. Au chips were cleaned by using plasma cleaning, employing Emitech (UK) plasma chamber. Plasma cleaning is a new powerful technique for removal of impurities and contaminants from surfaces by applying an energetic plasma created from gases like argon, oxygen or mixtures such

as air or hydrogen/nitrogen. It is advantageous comparing to common cleaning techniques, like inserting the sensor in active solutions such as piranha solution, because of simplicity, reproducibility and safety. The Emitech Plasma Chamber generates plasma based on radio frequency (RF) (usually 13.56 MHz) which is commonly used for cleaning surfaces. The cleaning is achieved by the ionic species created by the plasma, which are very reactive and can react with substances on the surface. The surface can be also cleaned by the ions produced by collision. After the reaction, the gaseous products are removed by vacuum. During the process very high temperatures are generated. The time and the power applied for the cleaning should be optimised since a prolonged or too intensive plasma cleaning can etch the surface. During the current work minimal time and power were used in order to obtain clean surface without etching the gold layer of sensor chips. Thus for cleaning of SIA Kit Au surfaces, oxygen plasma was applied for 3 min with a power of 40 W. The oxygen pressure in the chamber was set at 20mm Hg. The plasma chamber used for this work is shown on Fig. 3.4. After cleaning the chips from any present organic substances or contamination, the SIA Kit Au chips were immersed either in pure ethanol or directly in monomer solution (in ethanol) for SAM creation. Chips kept in pure ethanol were dried with nitrogen stream prior use. Ethanol was used in order to reduce the gold oxides, which are formed during the reaction of gold with oxygen plasma.

Fig. 3.4. *Plasma chamber (Emitech) with integrated Liquid and Gas Controllers (Horiba).*

SAMs were created on clean gold surfaces by immersing the chips in 10 ml thiol/OPA solution in DMF/ethanol (1:1) with 2:1 molar ratio thiol groups/OPA for 36 h. Triethylamine TEA (50 μl) was also added to the solution in order to facilitate thioacetal formation. The concentration of the thiol compounds tested was 0.1 M for di-thiol, 0.066 M for tri-thiol and 0.05 M for tetrathiol. OPA concentration was kept in all cases at 0.1 M. After immobilisation the gold surface was rinsed thoroughly with DMF/ethanol (1:1, HPLC grade), dried with nitrogen and the chips assembled on the holder. For the water soluble dithiol molecules, DTT and DMTZ, formation of SAMs was also performed and recorded on-line using Biacore by injecting 200 μl on a cleaned gold chip (2 injections × 100 μl, flow rate 5 μl min^{-1}) of DTT/OPA (0.066 M/0.033 M) or DMTZ/OPA (0.02 M/0.01 M) prepared in 50 mM $Na_2B_4O_7$, pH 9.0. In order to study the pH effect on SAM formation, DTT/OPA (0.066 M/0.033 M) solution was also prepared in PBS buffer pH 7.4 and the SAM was formed on-line (2 injections × 100 μl, flow rate 5 μl min^{-1}). All the solutions were purged with argon for five minutes to remove oxygen and were kept under inert atmosphere in order to

avoid oxidation. This on-line experiment could not be performed with other thiols (insoluble in water) as Biacore is not compatible with organic solvents. For the PETMP/OPA (the thiol with the best performance) the SAM formation was also studied using film bulk acoustic resonators (FBAR) with gold electrodes. FBAR are used for sensor applications. FBARs built as surface mounted resonators (SMR) consist of a piezoelectric layer, which is sandwiched between two electrodes. The energy losses, which result from acoustic waves propagation into the SI wafer, are prevented by an acoustic Bragg mirror.

These Si-integrated thin-film resonators are ordered as arrays on the chip. The frequency of resonance for the individual elements is usually in the GHz range. The binding of molecules on the resonator surface causes resonance detuned according to the mass attachment and it can be detected electronically. The biochemical functionatilisation creates the selectivity for individual elements. The thin-film arrays are advantegeous towards classical quartz crystal resonators due to the possibility to integrate the resonator array into a CMOS read-out circuit and the implementation of a multiplicity of elements on a very small area.

The FBAR experiments were performed in Siemens (Munich, Germany). During the experiment the SAM formation was recorded by pipetting amounts of solution directly to the sensor gold surface with a cell mounted on the FBAR, which was open on the top. The technique is described in more details in Tukkiniemi *et al.* (2009). In order to monitor the adsorption, firstly a baseline was recorded with 10 μl of DMF/ethanol (1:1) in the cell. After a stable baseline was reached, 90 μl of PETMP/OPA monomer solution was added at a concentration of 0.1 M. The cell was then closed with a lid to avoid evaporation of the solution.

3.2.3.2. SAM coated sensor surface characterisation

In order to determine the nature of the surface and get information about the success of modification the static water contact angle of the surface was determined with a CCD camera, supplied together with software by Spectra Source Equipment, model MCD400S (USA). Contact angle measurement (CA) is a simple and useful method for surface analysis, measuring surface energy and tension. The method is based on measuring angle between the tangent line from the droplet touching solid surface. If a liquid with well-known properties is applied, for instance water, the resulting interfacial tension can be used to determine the nature of the solid. If the tested surface is extremely hydrophilic a water droplet will spread completely (an effective contact angle of 0°). On 'average' hydrophilic surfaces exhibit contact angles usually between 10° and 30°. On the other hand on hydrophobic surfaces a large contact angle is observed (70° to 90°). In some cases, surfaces show water contact angles as high as 150° or even nearly 180°. On such surfaces, water droplets simply rest on the surface, without wetting to any significant extent. These surfaces can be characterised as superhydrophobic and can be obtained on fluorinated surfaces that have been appropriately micropatterned. In order to determine the composition of SAMs produced by the monomer with the best performance, X-ray photoelectron spectroscopy (XPS) was also used. The XPS experiments were performed in the School of Applied Sciences (Cranfield University). The measurements were carried out on a VG ESCA lab-Mark-2 X-ray Photoelectron Spectrometer (East Grinstead, UK). The X-ray gun was operated at 14 kV and 20 mA. Survey and high-resolution spectra were collected at 50 and 100 eV respectively, with Mg Kα radiation 1253.6 eV. Scans were obtained in the C1s, N1s, O1s, and S2p regions of the spectrum. The

decomposition of the XPS peaks into different components and the quantitative interpretation was performed after subtraction of the background using the Shirley method (Shirley, 1979).

3.2.3.3. Protein immobilisation on coated gold surface

The biomolecules used for evaluation of performance of SAM coated surfaces were bovine serum albumin (BSA), the enzymes trypsin, carbonic anhydrase, pepsin and the mouse monoclonal antibodies anti-PSA and anti-ST. Non-immunoactive mouse IgG was used as control on reference channel for experiments with PSA and ST cells detection. BSA was used for the initial assessment of the capacity of SAM surfaces to immobilise protein. Biacore C1 chips were used for comparison. C1 was initially cleaned with 2 min injection (20 µl, flow rate 10 µl min^{-1}) of NaOH 1 mM containing 0.03% Triton X-100. The chip was then activated by injecting 70 µl (flow rate 10 µl min^{-1}) of 0.2 M EDC/0.05 M NHS (Fagerstam et al., 1992). Typically, BSA and enzymes immobilisation on SAM coated SIA Au was carried out by injecting 75 µl of 100 µg ml^{-1} of protein solution in 0.01 M phosphate buffered saline (PBS), pH 7.4 with a flow rate 15 µl min^{-1}. For the study of pH effect on protein immobilisation on thiol SAMs, the proteins were diluted in the following buffers: 0.05 M acetate buffer pH 4.5 and pH 5.0, 0.1 M PBS pH 7.4 and Na-borate buffer 0.05 M pH 9.0. For immobilisation of biomolecules on C1 0.05 M acetate buffer, pH 5.0 was used instead of PBS. The stability of the immobilised biomolecules on SAMs modified surfaces was tested by injection of 10 µl of regeneration solution: 0.1%, sodium dodecyl sulphate (SDS) at a flow rate of 30 µl min^{-1}. For antibodies immobilisation (anti-ST, anti-PSA and mouse IgG) 75 µl of antibodies (50 µg ml^{-1}) diluted in PBS pH 7.4 were injected with flow rate of 15 µl min^{-1}. Running buffer was also PBS, pH 7.4. For

kinetic studies after antibodies immobilisation and blocking, the buffer was switched from PBS to PBS containing 0.005% surfactant (P20) in order to eliminate nonspecific binding and improve fitting to the Langmuir 1:1 binding model. After covalent coupling of the antibodies, remaining thioacetal groups were deactivated with 25 µl of 1 M ethanolamine hydrochloride at pH 8.5 followed by 2–4 injections of 30–50 µl of BSA (100 µg ml^{-1}). The evaluation of performance of antibodies immobilised was carried out by injecting the antigens PSA and ST cells into chip with corresponding antibodies. The antigens were diluted in PBS containing 0.005% of P20 and injected for 3–5 minutes with a flow rate of 20 µl min^{-1} and 5 µl min^{-1} for PSA and cells correspondingly. PSA and cells were injected at concentrations ranging from 3.3 to 832.5 nM and 10 - 10^{10} cells ml^{-1} respectively. The dissociation time for assessing the dissociation constant K_d was 120–180 s. Kinetic data was obtained using Biaevaluation software provided by Biacore. In all experiments a reference channel with immobilised mouse IgG was used in order to assess the binding specificity. In case of anti-PSA/PSA the surface was regenerated with a pulse of 5–30 µl of HCl/glycine 10 mM (pH 2.5) at a flow rate of 30 µl min^{-1}. For the surface with immobilised anti-ST/ST cells, regeneration was performed by injection of 10–90 µl of 1 mM NaOH, 30 µl min^{-1}.

3.3. Results and Discussion

3.3.1. Reactivity of thiols

The ability of the selected molecules (DTT, PETMP, TMPTMA, DMTZ, HDT, and NDT) to form a fluorescent isoindole after reaction with OPA and NH_4OH was initially assessed for the selection of the most promising thiol molecules. Under the experimental conditions the maximum fluorescent was obtained after 3 hours from

the addition of NH_4OH. In higher molecule/OPA concentrations the maximum signal was reached 5–10 minutes after addition of NH_4OH. Nearly no fluorescence was observed for the molecule/OPA solutions in the absence of primary amines. The stability of the thioacetal groups (thiol/OPA solutions) was also studied by recording the fluorescence signal during 6 weeks at regular intervals after addition of NH_4OH. The fluorescent intensities are illustrated in Table 3.1. The experiments were performed in triplicate. Table 3.1 shows that the molecules exhibiting the highest fluorescence and therefore the strongest ability to form the isoindole and bind primary amine were DTT and PETMP. This can be explained by the presence of electron withdrawing groups (–OH for DTT and ester groups for PETMP) in their structures (See Fig. 3.1) which increase the thiols acidity. As a result the thiol group is deprotonated more easily and the formation of thioacetal is facilitated. As expected, DTT showed limited stability because of its tendency to oxidise and decompose as the recorded fluorescence decreased considerably in 6 weeks. On the contrary, PETMP after 6 weeks in solution with OPA exhibited only a slight decrease in fluorescence. The fluorescence derived from the NDT/OPA and HDT/OPA reaction with NH_4OH was lower than the one obtained with DTT and PETMP due to the lack of electron withdrawing groups. TMPTMA and DMTZ did not show significant fluorescence possibly due to the formation of an unstable isoindole, which can undergo decomposition quickly. Another explanation for the lack of fluorescence could be the formation of non-fluorescent derivatives (Nakamura et al., 1982). Compounds with a long alkyl spacer between sulphur and oxygen and branching side-chains near thiol groups will yield isoindoles with increased stability (Jacobs et al., 1986; Stobaugh et al., 1983). Therefore PETMP with longer alkyl spacer between

sulphur and oxygen comparing to TMPTMA showed high fluorescence in contrast with TMPTMA, which did not produce any fluorescence.

Table 3.1. *The fluorescence emission at 430–440 nm after reaction of thiol/OPA solutions with NH_4OH in DMF/ethanol 1:1.*

Molecule	***Maximum recorded fluorescence after NH_4OH addition***						
	1st day	**1st week**	**2nd week**	**3rd week**	**4th week**	**5th week**	**6th week**
DTT	10.93 ± 0.34	10.47 ± 0.45	8.52 ± 0.54	7.55 ± 0.16	5.45 ± 0.24	3.18 ± 0.20	0.65 ± 0.06
HDT	8.09 ± 0.22	8.61 ± 0.28	7.53 ± 0.32	6.62 ± 0.35	6.04 ± 0.31	5.16 ± 0.18	4.98 ± 0.16
NDT	7.83 ± 0.40	7.99 ± 0.34	6.75 ± 0.20	6.89 ± 0.21	5.82 ± 0.20	4.08 ± 0.11	3.56 ± 0.21
PETMP	10.11 ± 0.36	9.93 ± 0.17	9.40 ± 0.50	9.40 ± 0.44	9.58 ± 0.35	9.31 ± 0.10	9.14 ± 0.16
DMTZ	---	---	---	---	---	---	---
TMPTMA	---	---	---	---	---	---	---

3.3.2. Characterisation of SAM coated sensor surfaces

SAMs were created on the gold surface by immersing the chips in thiol/OPA solutions. Immersing the gold chips into a polythiol solution without prior reaction with OPA can result in formation of a flat and disordered layer with restricted availability of thiol groups on the surface, due to adsorption (on the gold) of more than one thiol group. In order to prevent this and produce 'well oriented' SAMs with thiol terminating groups, Niklewski *et al.* (2004) suggested the protection of one thiol group by creating a thioester and deprotection after SAM formation on sensor surface. Accordingly the presence of OPA in the solution plays a double role. It forms thioacetal groups, which are necessary for amino coupling and secondly, thanks to thioacetal formation, prevents flat orientation of SAMs in the same way as thioesters

do. The characterisation of all the resulting SAMs was performed by measuring the water contact angle. Hydrophilic surfaces would be preferred to hydrophobic as usually this latter is affected by higher amount of unspecific protein adsorption. PETMP SAM, which exhibited the best performance in fluorescence experiments, was further characterised by XPS. Contact angle measurements for all the SAMs with and without the presence of OPA are shown in Table 3.2, where it can be seen that DTT produces the most hydrophilic surface. The addition of OPA leads to thioacetal formation, which has a significant impact on surface as the contact angle increases from 16.3 to 32.1. DMTZ creates relatively hydrophilic surfaces, but the addition of OPA results in a hydrophobic SAM with contact angle of 63.4°. HDT, NDT, PETMP and TMPTMA form a relatively hydrophobic gold surface and the addition of OPA did not show significant influence on contact angle values.

Table 3.2. *Contact angle measurements of surface with polythiol molecules with and without OPA. The measurements were repeated three times.*

	DTT	***DMTZ***	***HDT***	***NDT***	***PETMP***	***TMPTMA***
Contact angle thiol (^{0})	16.3 ± 0.1	33.1 ± 0.6	55.0 ± 3.1	63.3 ± 0.1	51.6± 1.2	48.6 ± 1.3
Contact angle (thiol + OPA) (^{0})	32.1 ± 1.5	63.4 ± 0.6	62.3 ± 0.1	63.3 ± 0.1	62.6± 0.7	53.1± 1.9

XPS analysis revealed the presence of sulphur on the coated sample (6.2%) but not on the bare gold surface. Monitoring the kinetics of the SAM formation on FBAR showed fast formation of a dissipative layer in the first few seconds, which is transformed into a less dissipative within a couple of minutes. Mass adsorption at saturation after 15 minutes was 31.6 ± 4.5 ng cm^{-2}, which corresponds to 4.3 × 10^{13} molecules cm^{-2}, and the recorded adsorption is illustrated in Fig. 3.5.

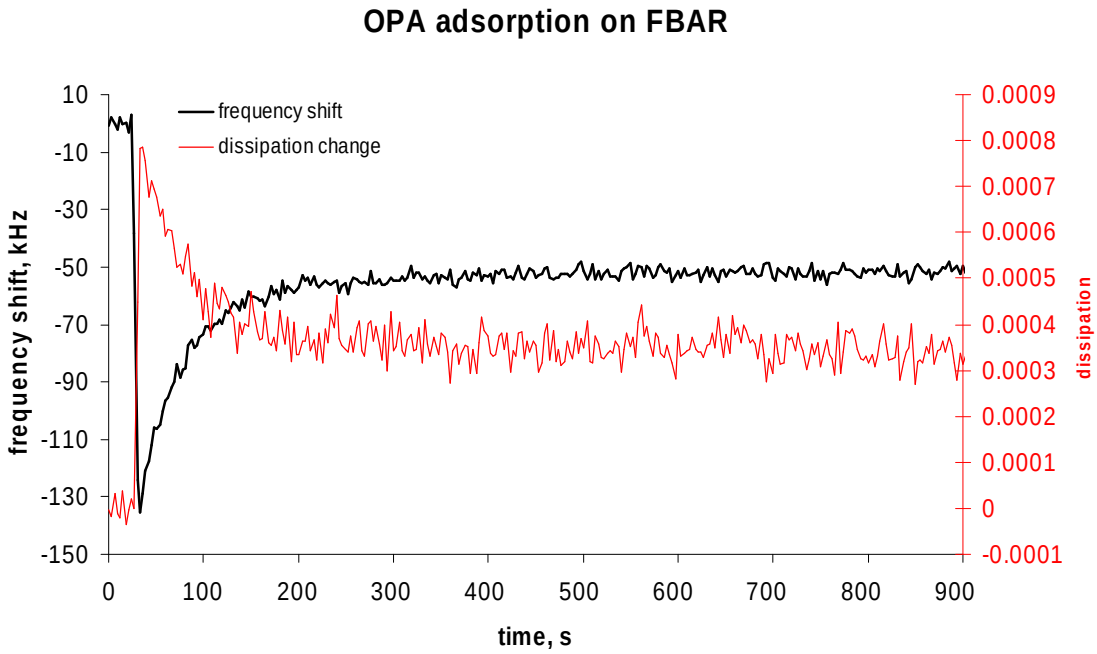


Figure 3.5. *SAM formation of PETMP/OPA on film bulk acoustic resonators with gold electrodes. The baseline was recorded in DMF/ethanol (1:1). After 20 seconds PETMP/OPA monomer solution was added.*

3.3.3. Evaluation of Monomer SAMs performance by SPR

3.3.3.1. Assessment of SAM ability to immobilise protein

The ability of the produced SAMs to immobilise proteins was studied using Biacore 3000. Initially BSA was used as a model biomolecule (100 µg ml^{-1}) and was immobilised as explained in the Material and Methods section. The experiment was repeated at least three times for each monomer and the affinity of immobilised BSA was assessed by injecting a solution of 0.1% SDS as described above. SDS is a surfactant that reduces hydrophobic interactions and consequently can wash away proteins loosely attached to the surface through hydrophobic interactions. The obtained results are shown in Table 3.3. In addition, in order to confirm that BSA was immobilised covalently through the reaction reported in Fig. 2.13, the immobilisation of BSA on SAMs was also performed without the use of OPA. The results are shown in Table 3.4.

Table 3.3. *Biacore responses to BSA immobilised on SAMs produced by different molecules. The standard deviation (STD) within the chip and between different chips is also reported. The last column shows percentage of material removed after injection of SDS 0.1%.*

Thiol	***n****	***Immobilised BSA (RU)***	***CV within each chip (%).***	***% removed BSAafter injection SDS 0.1%***
DTT	6	1613.1 ± 339.3	6.1	4.9 ± 0.4
HDT	3	1203.3 ± 93.6	4.9	26.4 ± 3.7
MDT	3	1095.4 ± 153.2	9.4	6.5 ± 1.3
NDT	3	1194.1 ± 287.3	2.9	9.7 ± 1.6
PETMP	6	1487.0 ± 70.1	2.2	4.3 ± 2.2
TMPTMA	4	1327.6 ± 133.8	3.4	9.1 ± 5.5

* Number of chips used.

Table 3.4. *Biacore responses to BSA immobilised on SAMs produced by different molecules without applying OPA. The standard deviation (STD) was calculated for four measurements. The last column shows material removed after injection SDS 0.1%. The STD was calculated for a set of 3 measurements.*

Thiol SAM	***Immobilised BSA (RU)***	***% Removed BSA after injection SDS (0.1%)***
DTT	621.4 ± 199.5	23.7 ± 4.3
HDT	411.2 ± 42.3	46.3 ± 17.4
DMTZ	664.0 ± 139.7	10.2 ± 2.7
NDT	531.3 ± 218.6	68.5 ± 12.4
PETMP	576.5 ± 86.9	33.7 ± 5.2
TMPTMA	691.4 ± 221.4	39.1 ± 12.4

By comparing Tables 3.3 and 3.4 it is clearly demonstrated that the addition of OPA during SAM formation increases the binding capacity, since there is a significant higher amount of immobilised protein on sensor surfaces in presence of OPA

comparing with SAMs without OPA. More importantly OPA stabilises the immobilised material on the surface as only a small amount of immobilised BSA was washed away after injecting SDS (0.1%). This is a strong indication that biomolecules are immobilised on the surface mainly through the strong covalent bond and not through Van der Waals or hydrogen interactions . Part of BSA can also be attached directly on bare gold, which could be presented due to inadequate surface coverage from SAMS and holes in monolayer. The differences in values between Table 3,3, and 3.4 also show that if this is happening it only accounts for a minimal amount of BSA immobilised.

As it is illustrated in Table 3.4, DTT creates SAMs with high protein capacity. The STD between DTT chips is however high and this is probably due to the tendency of DTT to get oxidised easily (sensitive to air) and to its hygroscopic characteristic. These properties influence the reproducibility of the results, since oxidised thiol groups yield to disulphides formation, which are not able to participate in any further reaction to form thioacetals. Regarding the hygroscopic behaviour, most likely the amount of DTT weighted each time varied according to the moisture absorption. Decomposition of DTT takes place preferentially at pHs higher than 8.3 (pKa of thiol is 8.3), as negatively charged thiol groups are more reactive (Cleland, 1964). It is also crucial to keep the solution of DTT/OPA under inert atmosphere in order to prevent the fast oxidation and use DTT from fresh opened bottles. It was, in fact, observed that when DTT was used for long time (over 1 month) the binding of BSA was 50-75% less than the amount obtained in earlier experiments performed with fresh DTT. Despite these disadvantages DTT forms SAM with high surface capacity, higher than, for example, the other water soluble monomer, DMTZ (see Table 3.4). DTT STD within chips is much lower, which proves that using fresh DTT, exposed as briefly as

possible to atmospheric conditions, can lead to better reproducibility. The results reported in Table 3.3 and 3.4, showing capacity of DTT and DMTZ, are those obtained by producing SAMs on-line on the Biacore. For DTT the on-line value, was higher than the one recorded when SAMs formation was performed by immersing the chip in the monomer solution for one day (1287.5 ± 211.8 RU). Probably this is still due to the limited stability of the molecule. In case of DMTZ there was not significant difference (within the STD range) for protein capacity recorded when the SAM formation was performed on-line or by immersing the chip in solution. The other four monomers are soluble only in organic solvents and on-line SAMs formation was not possible, because of the incompatibility between Biacore and organics solvents. Nonanedithiol (NDT) and Hexanedithiol (HDT) are compounds with similar structures, with the only difference being in the number of carbons atoms present in their carbon chains (see Fig. 3.1). Fluorescence results have illustrated (see Table 3.1) that HDT performed better than NDT in producing isoindoles in solution. However SAMs of NDT performed better than those of HDT in binding biomolecules. The protein capacity of the two SAMs is similar, but the stability of immobilised protein on HDT SAMs is significantly lower, since 26.4% of immobilised BSA was removed after SDS injection. This can be explained by the fact that a longer alkane chain (as on NDT) provides more Van der Waals interactions between neighbouring molecules immobilised on surface, allowing formation on the gold surface of an ordered and standing thiol layer (Bain *et al.*, 1989; Holmes-Farley *et al*., 1988), which would be more capable of immobilising biomolecules. HDT most likely produces a SAM, which is not as ordered and therefore not as able to bind proteins. The disadvantage of applying NDT for SAMs formation is, however, similar to DTT one and it is related to the stability of the compound after exposure to atmospheric conditions. In fact

Table 3.3 shows that, also for NDT, as for DTT, the STD is high for measurements performed with different chips and is low within the same chips. Chips where SAMs were made with fresh NDT showed higher capacity for protein immobilisation with relatively high affinity comparing to SAMs formed by 'old' NDT.

TMPTMA and PETMP are molecules containing three and four thiol groups respectively. PETMP SAMs showed higher surface capacity for protein immobilization with 1487.0 RU, than TMPTMA, which produced a signal of 1327.6 RU. Additionally, as illustrated in Table 3.4 PETMP layer exhibited higher stability as only 4.3% of the immobilised BSA was removed after washing with SDS, whereas 9.1% was removed from TMPTMA layer. Another important characteristic shown by the experiments was that protein deposition on the PETMP layer was not affected by the freshness of the monomer used for the SAMs. In fact comparable results were obtained either using PETMP from a freshly opened bottle or from one stored for some time. The high stability of this monomer was also proven by the low CV (4.7%) for protein immobilisation calculated using six different chips over a 3 months period. On the contrary TMPTMA seemed to be affected by stability problems especially after exposure to atmospheric conditions. In fact, for example, the TMPTMA and OPA solution, prepared for the SAM formation, was initially colourless, but was visibly becoming yellow and later brownish in relatively short time. This change in colour may most likely be related to thioacetals degradation and reformation of OPA or its derivative. Despite the fact that TMPTMA and DMTZ did not show fluorescence in solution the observed protein immobilisation can be due to other reactions of thioacetals with amino groups, which results in non-fluorescent products (Nakamura et al., 1982).

In conclusion SPR experiments are generally in agreement with fluorescence studies. Both have shown that the monomers with highest performance for amine coupling are DTT and PETMP. Both sets of results also have shown the same stability profile. Also the fluorescence experiments have shown PETMP as one of the most stable monomer and DTT one of the least stable. Due to its high reproducibility, stability and good affinity, PETMP was selected for further studies. DTT was also considered for further studies due to the hydrophilicity of the produced SAM, which should have minimal amount of unspecific binding.

3.3.3.2. Effect of pH on proteins immobilisation

The monolayer formed by DTT/OPA was applied to study the pH effect during SAM formation by investigating its protein capacity. The effect of pH was critical during DTT/ OPA SAM formation on gold. When attachment of DTT/OPA was performed in PBS pH 7.4 instead of borate buffer pH 9.0, a reduction (40-60 %) of BSA binding was observed in the following step. This was expected because the reaction between OPA and DTT to form thioacetal is facilitated by pH higher than 8.0, where the thiols are mainly negatively charged. The pH effect during protein immobilisation was also studied by using DTT and PETMP SAMs. It is well known that protein immobilisation is highly dependent on pH (Branden and Tooze, 1999). In this work effect of pH in a range of 4.5–9.0 (0.05 M acetate buffer pH 4.5, 0.05 M acetate buffer pH 5.0, 0.1 M PBS pH 7.4 and 0.05 M Borate buffer pH 9.0) on protein immobilisation was studied using BSA (100 μg ml^{-1}) as a model protein. The results are shown in Fig. 3.6. The experiments were carried out in triplicate. The highest immobilisation was achieved when BSA was immobilised using PBS buffer pH 7.4 and borate buffer pH 9.0 for both PETMP and DTT SAMs. Even though Figure 3.6 shows that highest immobilisation of BSA was obtained when performed using 0.05

M $Na_2B_4O_7$, pH 9.0, the difference with the immobilisation performed in PBS, pH 7.4 is not significant. The decrement of protein binding in acidic conditions could be due to an inhibition of the isoindole formation at low pH. These findings show that the crucial step, which requires basic pH, is the hemithioacetal formation. Afterwards, the immobilisation of biomolecules can be done at pH 7.4, which would be ideal to develop clinical sensors, as it resembles physiological conditions.

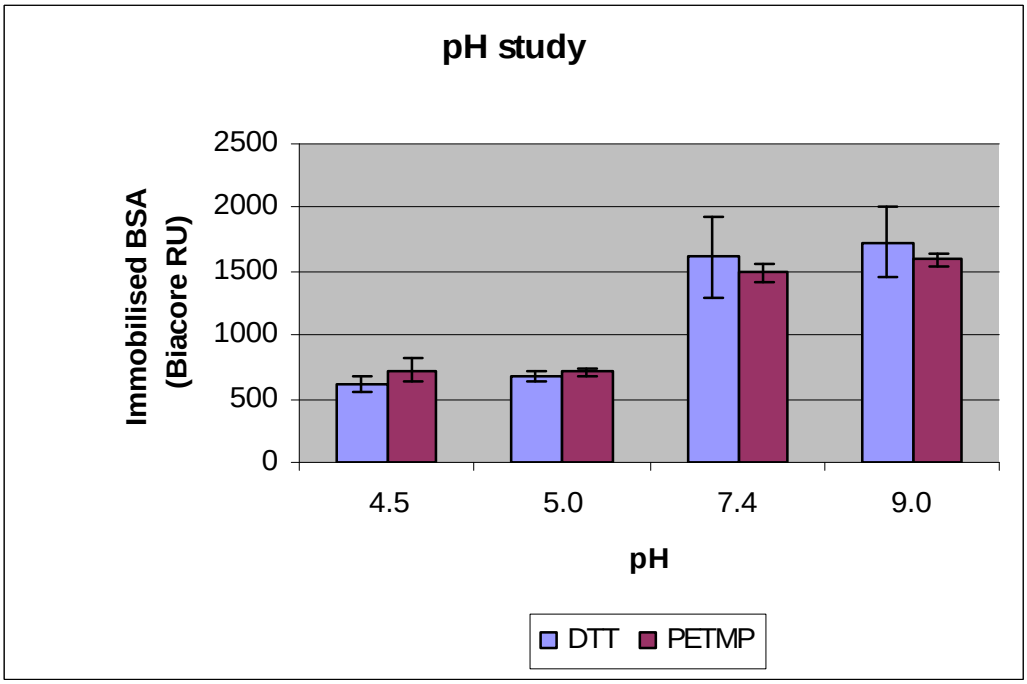


Fig. 3.6. *Influence of pH on the immobilisation of BSA (100 μg ml^{-1}) onto DTT/OPA and PETMP/OPA SAM. The error bars represent the STD of Biacore responses which was calculated for set of 3 measurements.*

3.3.3.3. Application of PETMP/OPA SAM and comparison with Biacore chip C1

In order to assess the performance of newly developed SAMs the comparison with commercially available Biacore chip C1 was imperative. Thus kinetic analysis of PSA binding on immobilised anti-PSA antibody and detection of large analyte as *Salmonella typhimurium* cells by anti-Salmonella antibody were performed both on PETMP SAM and C1.

i) Kinetic analysis of PSA/anti-PSA monoclonal antibody interaction

Flat sensor surface modifications are useful for the determination of kinetic constants and evaluation of affinity of binding reactions. Application of flat surfaces with low volume and restricted surface capacity is important, due to the fact that in this condition mass transport limitation has a minor impact on the resulting sensogram (Önell and Andersson, 2005). In order to minimise the limitation of mass transport, when a sensor chip with high surface capacity is applied for kinetic studies, the immobilisation level of the ligand should be kept low (500–2000 RU) (Katsamba *et al.*, 2006). Functionalisation providing low volume surfaces can also result in less nonspecific binding (due to limited charge attractions and hydrophobic interactions), which, if it is not eliminated, can have a prominent effect on the calculation of kinetic constants. Also Biacore recommends low capacity sensor surfaces for kinetic studies (Biaevaluation Handbook). As it has been previously mentioned during the pH study, the highest immobilisation was achieved when BSA was immobilised in PBS buffer pH 7.4 and borate buffer pH 9.0 (Fig. 3.6). Consequently the immobilisation at pH 7.4 was selected for further studies on PETMP/OPA SAM, since it resembles physiological conditions, while still allowing for good protein immobilisation. DTT/OPA SAMs were also considered for kinetic experiments, but unexpectedly they did not show satisfactory results because of the presence of high non specific binding. The reason for this could be due either to the short carbon chain of DTT or to the insufficient time used for SAM formation. As it is reported in literature, the presence of long carbon chain favours a highly ordered standing orientation of thiols (Bain *et al.*, 1989; Holmes-Farley *et al*., 1988) thanks to Van der Waals interactions between carbon chains. Regarding the time employed for SAM formation, it is well known that the interaction between sulphur and gold is very fast and spontaneous, but the

formation of a highly ordered SAMs requires from few hours to one day (Dubois and Nuzzo, 1992). In this study DTT/OPA SAM was formed on-line on Biacore with a total immobilisation time of 40 minutes, which might not be long enough to produce an ordered thiol layer. Comparative studies between PETMP/OPA SAMs and Biacore C1 sensor chips were performed with monoclonal anti-PSA antibody. For the kinetic study, of PSA-anti PSA monoclonal antibodies (mAb) were immobilised on PETMP /OPA SAMs and Biacore C1 sensor chips with amino-coupling chemistry by applying different reactions as it was previously described. Relatively high amounts of mAb were immobilised on both sensors in order to achieve a complete coverage (saturation) and homogeneity of the surface. Immobilisation of mAb on PETMP-OPA and C1 produced a Biacore signal of 4572.4 ± 105.6 and 3145.1 ± 85.4 resonance units (RU) respectively. Each experiment was performed in triplicate. The kinetic constants, determined with the Biaevaluation software, provided by Biacore, were k_a (the rate of formation of new complexes), k_d (the rate of complex dissociation) and K_D (equilibrium dissociation constant). In general for a 1:1 Langmuir binding model, kinetic constants values between 10^4 to 10^7 $M^{-1}s^{-1}$ for k_a and 10^{-4} to 10^{-1} s^{-1} for k_d can be determined with high confidence. Higher or lower kinetic constants might be affected by errors produced either during experiments or during the fitting of the data (Önell and Andersson, 2005). In this study PSA in varying concentrations (3.33–166.5 nM for PETMP/OPA SAMs and 33.3–832.5 nM for C1) were injected on both surfaces for each fitting, Fig. 3.7. The concentrations of PSA used on C1 surface were higher because, due to lower surface affinity, low concentrations resulted in inadequate responses. Very low concentrations, as well as high concentrations were avoided in the attempt to obtain more precise fittings. In fact at low concentrations the noise contribution may be significant and at high concentrations other interactions can

lead to deviations from the 1:1 Langmuir model. The sensograms obtained with both sensor chips fitted well the 1:1 Langmuir binding model (Fig. 3.7). The parameter x^2, which is used to measure the accuracy of the fittings, had a value of less than 2, which indicates excellent fitting (Biaevaluation Handbook, 2004). Specifically the x^2 values for PETMP-OPA and C1 sensor were 0.341 and 1.54 respectively (Table 3.5). Statistical information on the data is given by the *T*-value, which is the relative measure of the standard error. *T*-value is determined by dividing the value of the parameter (in this case k_a and k_d) by the standard error. A *T*-value higher than 10, which corresponds to less than 10% standard error, is considered satisfactory (Biaevaluation Handbook; Önell and Andersson, 2005).

Table 3.5. *The calculated kinetic constants for anti-PSA/PSA interaction on C1 and on PETMP-OPA SAM.*

Surface	k_a $(10^4 M^{-1}s^{-1})$	$T(k_a)$	k_d $(10^{-3} s^{-1})$	$T(k_d)$	K_D (nM)	x^2
PETMP-OPA	4.52	35.4	4.77	10.2	106	1.54
Biacore C1	5.36	49.1	4.19	18.1	79.3	0.341

The values of K_D obtained here (Table 3.5) demonstrate relatively low affinity for PSA antigen if compared to previous studies where different monoclonal Ab showed much higher affinity with a K_D of only 1 nM (Katsamba et al., 2006). The differences of the association constant k_a and dissociation constant k_d values calculated for the two surfaces for the same antibody–antigen reaction are satisfactory since deviations for kinetic constants determination of 15–20% are acceptable (Myszka et al., 1998; Katsamba et al., 2006; Önell and Andersson, 2005). The similarity between kinetic

values obtained on PETMP-OPA and C1 surfaces shows that both can be equally used as a low capacity flat surface sensor for kinetic studies.

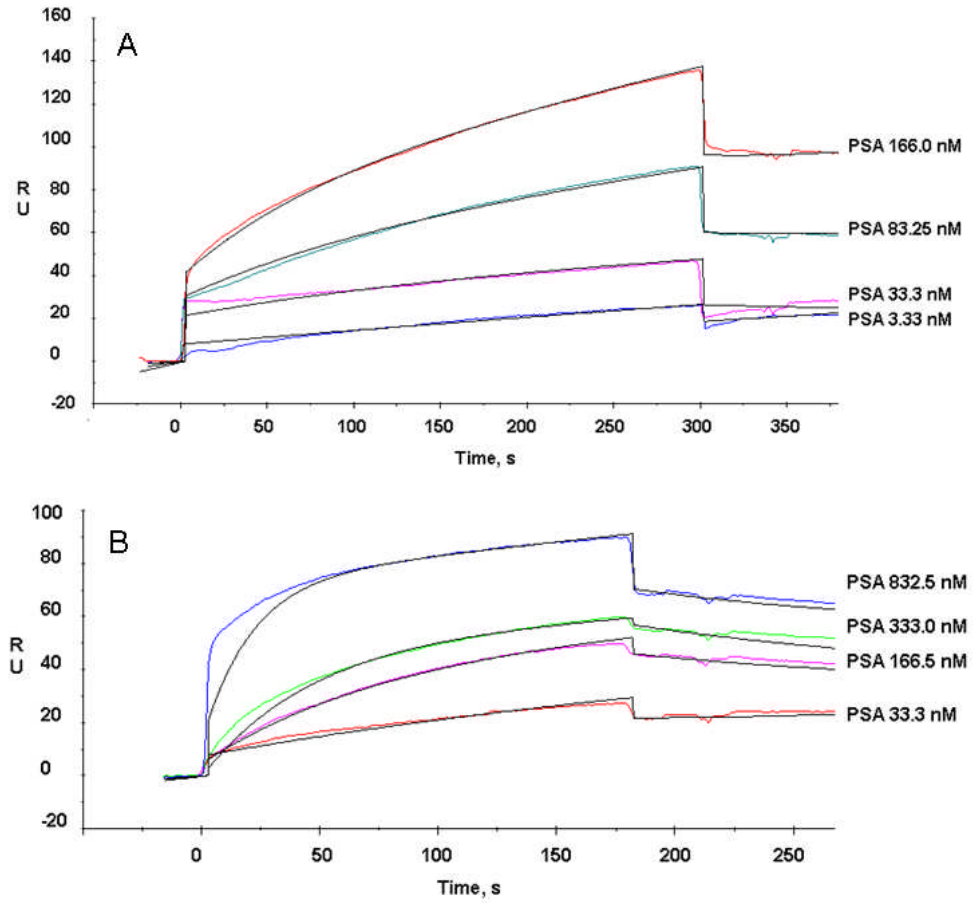


Fig. 3.7. *Kinetic studies of PSA binding to the immobilised antibody on modified PETMP/OPA SPR surface (A) and C1 (B) The grey line shows the fitting according to 1:1 Langmuir binding. PSA concentrations were 3.33, 33.3, 83.25, 166.5 nM.*

ii) Detection of Salmonella Typhimurium cells

One of the main applications of flat sensor functionalisation in SPR is the detection of large analytes like cells or viruses. The reason is that, since the evanescent wave useful for SPR measurements is only 300 nm from the sensor surface, the use of chips as CM5 with high capacity layers of around 100 nm, makes detection of large molecules challenging. In fact, large analytes have shown poor responses on such

surfaces as the sensitivity decreases exponentially with the distance from the sensor surface (Nagata and Handa, 2000). Another issue is that, because of their dimensions, only a restricted amount of large analytes can be immobilised on the surface. Biacore Sensor Surface Handbook itself reports that detection of large analytes is favoured on flat sensor surfaces like C1. Hence the newly developed SAM and C1 were both applied for *Salmonella* typhimurium cells detection and the results compared. For this application anti-Salmonella typhimurium antibodies (anti-ST) were immobilised both on PETMP-OPA SAM and C1 chips as described in Materials and Methods section. A reference channel with immobilised mouse IgG was used in order to assess binding speicificity. The antibodies immobilisations produced Biacore signals of 2461.4 ± 71.4 RU and 1543.4 ± 89.0 RU for PETMP SAM and C1 respectively. Standard deviations (STD) were calculated using three separate immobilisations. The newly developed surface showed higher capacity than C1 for antibodies immobilisation. After blocking with TEA and BSA, several cells dilutions prepared in PBS buffer were injected both on working and reference channel with a slow flow rate
(5 μl min^{-1}) in order to avoid blockage of the injection system. The detection of cells in real time is illustrated in Fig. 3.8a and 3.8b.

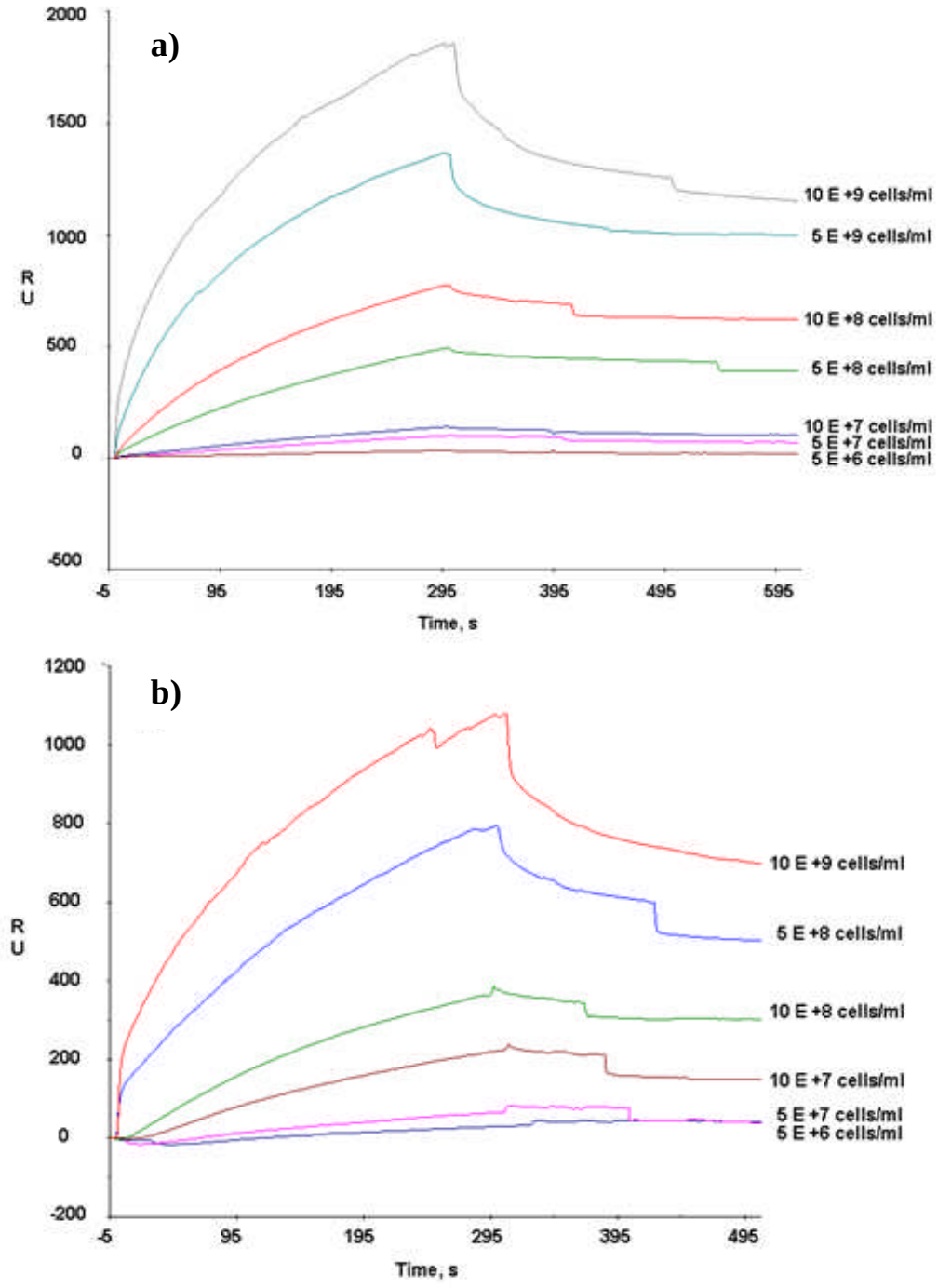


Fig. 3.8. *SPR sensogram showing real time detection of ST cells in various concentrations (cells ml^{-1}) on PETMP-OPA surface (3.8a) and C1 (3.8b).*

The resulting calibration curves are reported in Fig. 3.9. The curves were calculated by subtracting the response recorded on reference channel for corresponding

concentrations (non specific binding). The nonspecific binding to both SAM and C1 was determined by injecting the same cell concentrations on a reference channel containing non specific antibody. The nonspecific binding was in general higher for the PETMP-OPA SAMs especially when the two highest cells concentrations were applied. In fact nearly 10% of non specific binding was present when 5×10^8 and 1 x 10^9 cells ml^{-1} were injected. On the contrary the non specific binding recorded on C1 for the same concentration was negligible. The higher amount of non specific binding to PETMP-OPA SAM as compared to C1 is possibly related with the hydrophobicity of the surface.

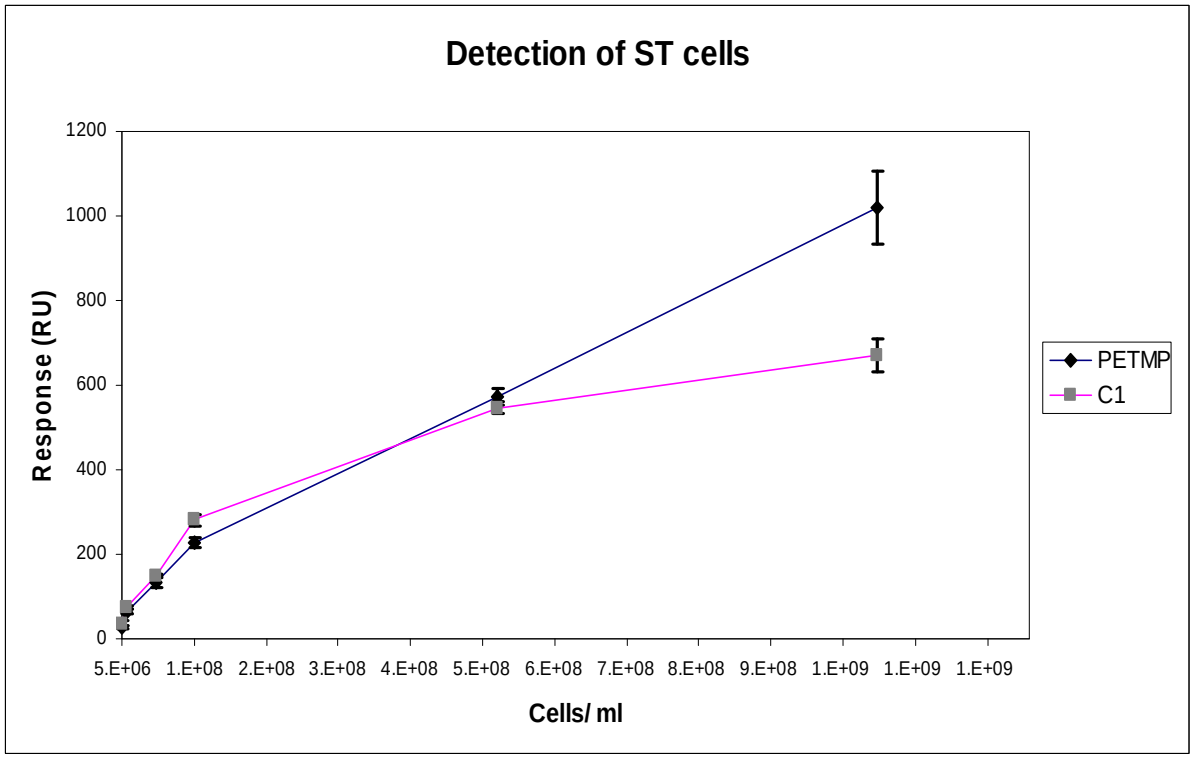


Fig. 3.9. *Calibration curve for ST cells detection obtained using monoclonal anti-ST antibody immobilised on PTEMP-OPA and C1 surface. Error bars represent the STD of Biacore responses (RU) for each concentration and it was calculated from a set of 3 measurements.*

The higher response observed in Fig. 3.9 for PETMP SAM surface, when high concentrations of cells were injected could be due to the higher amount of

immobilised Ab. In fact as it is shown in the Fig. 3.9 detection on C1 chip exhibits earlier saturation than PETMP SAM. At 10^9 cells ml^{-1} the calibration on C1 shows significant saturation, whereas on PETMP SAM this concentration is still in linear range. The lowest concentration giving at least three times higher response on monoclonal antibody surface than on reference channel was considered as limit of detection. Detection limits of 5 $x10^6$ cells ml^{-1} were obtained for PETMP SAM and C1 sensor chip. The sensitivity and consequently the detection limit for immunoassays is highly dependent on the affinity of the applied antibody. A lower detection limit (1.25×10^5 cells ml^{-1}) for the same bacteria was for example reported by Mazumdar *et al*. (2007) by using a sandwich assay. The highest sensitivity could be due both to the quality of the antibody used by Mazumdar or by the detection system (sandwich assay instead of direct testing).

3.3.4. Study of pI effect for proteins immobilisation on PETMP SAM and C1

A challenging aspect during sensor fabrication is the immobilisation of proteins with different isoelectric points (p*I*) on the same substrate. For these experiments proteins with p*I* ranging from 1 to 10 were tested on PETMP chips and also on C1 for comparison. Pepsin (p*I* 1.0), carbonic anhydrase (p*I* 6.5), trypsin (p*I* 10) and BSA (p*I* 4.5–5.5) were immobilised on the chips and the response monitored on Biacore. The results are reported on Table 3.6. All the experiments were performed in triplicates.

Table 3.6. *The immobilisation of proteins with different pI on PETMP and C1 chips.*

Protein	***pI***	***PETMP-OPA coated surface (RU)[a]***	***C1 (RU) (%)[a]***
Pepsin	1.0	1102 .4 ± 32.7	46.1 ± 9.1
Carbonic anhydrase	6.5	1374.7 ± 56.6	595.2 ± 32.1
Trypsin	10	1581.1 ± 92.4	155.1 ± 21.2
BSA	4.5-5.5	1487.0 ± 61.5	940.9 ± 58.1

[a] Standard deviation was calculated from a set of three experiments.

As suggested by Biacore, the pH of the buffer used for immobilisation on C1 chips was 5.0, as this produces maximum attachment of positively charged protein to the negatively charged carboxylic group present on the sensor surface. An acidic pH is in fact required to immobilise positively charged proteins. The use of a pH lower than 5.0 is however not advisable as it might inhibit peptide bond formation. This means that proteins with a p*I* lower than 5.0–5.5 will attach to C1 surface with difficulties. Table 3.6 shows that the novel SAM possesses reasonably capacity for all the tested proteins. The most significant advantage as shown is the possibility to immobilise pepsin, which is a protein with a very low p*I* (p*I* = 1.0), onto PETMP SAM, with nearly no attachment onto the C1 (1102.4 RU on PETMP and 46.1 RU on C1). This is most likely due the electrostatic repulsion between the negative charges present on the protein at pH 5.0 and the negatively charged carboxylic groups present on C1 layer. On the other hand, the application of amino coupling SAM produces a neutral surface, which lacks significant electrostatic charges. Hence, negatively charged proteins can approach the surface in close proximity and immobilised easily onto the SAMs. Another advantage resulting from the absence of charges on the developed PETMP SAM surface is the opportunity to detect charged analytes by avoiding the

non-specific binding caused by electrostatic attractions of interfering compounds with similar charges. In conclusion the novel polythiol SAMs reported here seems to be generic layers capable of immobilising proteins regardless of their chemical properties such as p*I*.

3.4. Conclusions

The work presented in this chapter has described the development of a novel, low cost, fast and simple method for polythiol SAMs formation on gold surface, which enables immobilisation of proteins through amino coupling using a chemistry, alternative to peptide bond formation. The amino coupling is based on the well known reaction of thioacetals, formed by reaction of thiols groups with aldehydes, with primary amino groups resulting into the formation of fluorescent isoindoles. On the contrary of many methods used for amino coupling, the thioacetal groups are able to bind amino containing substances without any activation step. Several polythiols monomers were tested for their ability to form polythioacetals self-assembled monolayers (SAMs) and their capability to immobilise biomolecules. Among those tested, PETMP SAMs showed the highest stability and surface capacity for proteins immobilisation. PETMP SAMs were utilised for kinetic studies of anti PSA- PSA interaction and for detection of *Salmonella Typhimurium* cells. The performance of the novel SAMs was then compared with the commercially available Biacore C1 chips. Both in kinetic study and in the detection of cell the results obtained with PETMP SAMs were better or comparable with those achieved by using the C1 chips. In fact the kinetic constants were $k_a = 4.52 \times 10^4\ M^{-1}s^{-1}$, $k_d = 4.77 \times 10^{-3}\ s^{-1}$ and $k_a = 5.36 \times 10^4\ M^{-1}s^{-1}$, $k_d = 4.19 \times 10^{-3}\ s^{-1}$ for PETMP SAM and C1 correspondingly. Regarding Salmonella Typhimurium cells detection, a similar LOD of 5×10^6 cells

ml^{-1} was obtained for both surfaces. However whereas PETMP SAM was saturated at 10^9 cells ml^{-1}, C1 reached saturation already at 5×10^8 cells ml^{-1}. Another remarkable advantage of PETMP SAMs is their ability to immobilise successfully any protein regardless of their chemical properties such as the isoelectric point. Additionally the low cost involved in polythiol SAMs formation and the simple and general protocol required for biomolecules immobilisation can be considered a significant advantages over most of the commercially available functionalised trasnducers used nowadays for the development of biosensors. Therefore all the benefits and findings, reported here, make the newly developed polythiol SAMs very promising for future biosensors applications.

Despite all the reported benefits of applying flat sensor surfaces, the inability to achieve a detection limit for PSA in buffer low enough for practical applications created the demand for the development of a 3-D surface functionalisation. A surface modified with a 3-D polymer should possess higher capacity for biomolecules and consequently should allow achievement of higher sensitivity producing immunosensors useful for testing real clinical samples.

CHAPTER 4

POLYMER DEVELOPMENT –

SPR APPLICATIONS

4.1. Introduction

In this chapter the development and synthesis of a reactive polymer suitable for covalent immobilisation of proteins and nucleic acids onto sensor surfaces is reported. We have proceeded to the synthesis of polymers in order to increase the sensitivity of immunoassays, since it has been demonstrated in the past that the use of flexible porous and non-porous polymer films, consisting of various molecular weight polymer fractions or different length spacer arms, to attach biomolecules produces more sensitive assays than homogeneous flat surfaces (Masson et al., 2005). This can be attributed to an improvement of protein diffusion in the polymer matrix, together with partial protection of protein structure from unfolding processes. Rigid or solid surfaces often cause irreversible denaturation of the bound proteins (Su et al., 1998). Thus particular care is taken to protect the immobilised receptors/ligands from denaturing processes during or post immobilisation (Butler, 2000). The main objective of our work was to achieve successful detection of PSA in the range of analytical interest for clinical applications (1-10 ng ml^{-1}).

The requirement of creating a novel matrix with low level of non specific binding during immunoassay development with, therefore, potential for clinical applications was taken into consideration during polymer synthesis. This is because real samples such as plasma serum are very complex and contain thousands of different molecules (Kusnezow and Hoheisel, 2003). A low level of nonspecific binding is usually achieved by inclusion of polar molecules such as polysaccharides or polyethylene glycol derivatives into the immobilisation matrix (Masson et al., 2005).

This novel polymer developed during this work contains thiol derivatives (disulphide or thioethers) that promote self-assembling onto a metal transducer surface. The biomolecules immobilisation is based on the reaction between primary amines and

thioacetal groups contained in the polymer matrix (Simons and Johnson, 1978; Piletska *et al.*, 2001), the same chemistry described in previous chapter for attachement on polythiols SAMs (Fig. 2.13).

The product of the reaction is a fluorescence isoindole (Fig. 2.13 and Fig. 4.1). As mentioned before, this reaction can take place without any activation, which makes this novel polymer suitable for sensor/ array fabrication. In addition the flexibility of the polymeric tri-dimensional (3D) network allows a high density of receptor immobilisation, while ensuring the availability of a high percentage of binding sites. For demonstration purposes the 3D polymer was self-assembled onto gold Biacore chips (SIA Kit Au) and the entire testing was performed using Biacore 3000. Nevertherless there is potential to use this type of material on any noble metal transducer and not only for optical biosensors. Initially the ability of the polymer surface to bind proteins/receptors was tested by immobilising different ligands such as bovine serum albumin (BSA), three enzymes with different isoelectric points, one type of polyclonal antibodies (anti-microcystin-LR) and one type of monoclonal antibodies (anti-prostate specific antigen or anti-PSA). The results obtained for the immobilisation of BSA and the three enzymes on the polymer were compared with those obtained immobilising the same proteins on commercially available pre-functionalised Biacore chips (carboxydextran CM3 and CM5). Finally the quality of the receptor immobilisation was evaluated by binding the antigens (microcystin-LR and PSA) to the corresponding antibodies immobilised onto the novel polymer. The encouraging results observed during this work make this new polymeric matrix very promising for the development of low-cost, easy to prepare and sensitive biosensors. A schematic representation of the modification of gold sensor surface (SIA Kit Au) and the immobilisation procedure is illustrated in Fig. 4.1. All the specific binding

reactions (e.g. antibody/antigen) reported in this chapter were performed in model solutions (buffer solutions). Further immunoassay developments necessary to be able to detect PSA in serum and consequently in clinical samples is described in the next chapter, Chapter 5.

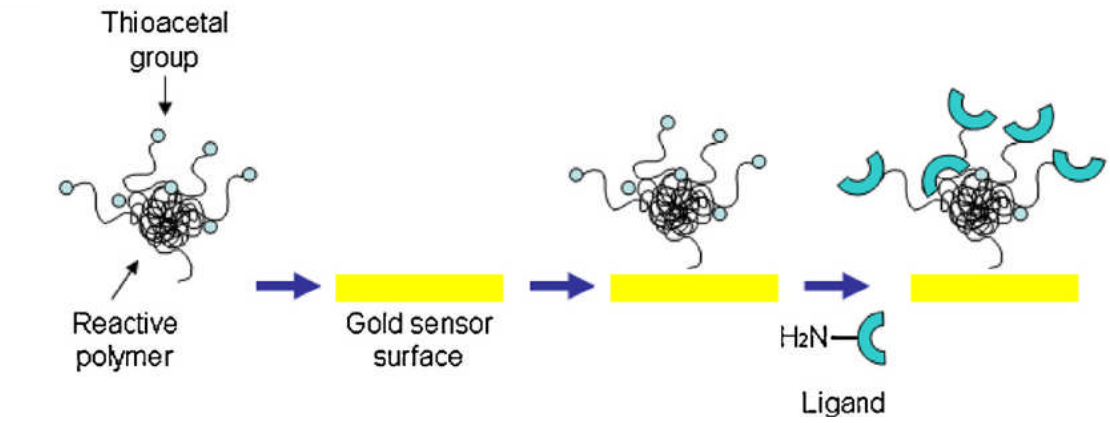


Fig. 4.1. *Schematic representation of biomolecule immobilisation on the tri-dimensional polymer-coated gold surface.*

4.2. Materials and Methods

4.2.1. Reagents

Most compounds were obtained from commercial distributors and were of analytical or HPLC grade. 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), Triethylamine, Azo – isobutyronitrile (AIBN), 2-Hydroxyethyl methacrylate (2-HEM), ethylene glycol dimethacrylate (EGDMA), Methyl Methacrylate (MMA), 1,1,1-tris (hydroxymethyl)propan trimethacrylate (TRIM), 2-benzyl-2 (dimethylamino)-4-morpholino-butyrophenone (BDMB), bovine serum albumin (BSA, lyophilised powder), anti-sheep secondary antibody (anti-sheep Ab), Calf Thymus DNA, IgG from bovine serum, the enzymes trypsin (lyophilised powder, from bovine pancreas), carbonic anhydrase (electrophoretically purified, dialysed and lyophilised), pepsin

(lyophilised powder from porcine gastric mucosa) were purchased from Sigma–Aldrich (UK). Sheep polyclonal anti-microcystin-LR antibody (anti-microcystin-LR Ab) was provided by Prof. Hennion from the Department of Environmental and Analytical Chemistry in Paris (Rivasseau and Hennion, 1999). Microcystin-LR was from Alexis (Switzerland). Anti-PSA mouse monoclonal Ab and PSA were purchased from AbD-Serotec (UK) and Alpha Diagnostics (UK) respectively. Allyl thiol (AT), *N*,*N* - bis(acryloyl)cystamine (BAC), *o*-phthaldialdehyde (OPA) were purchased from Fluka (UK). Ethanolamine, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS), sodium dodecyl sulphate (SDS) solution (10%), SIA Kit Au, CM5 and CM3 chips were purchased from Biacore (Sweden).

The water was purified by Milli-Q water system (Millipore, Bedford, MA, USA) and all the reagents used for Biacore experiments were filtered using a 0.22 μm filter from Phenomenex® (UK).

The solvents Acetonitrile (HPLC), Tetrahydrofurane THF for HPLC and Methanol were from Acros Organics (UK). N,N-Dimethylformamide was purchased from BDH; Acetone for HPLC and Ethanol were from Fisher scientific.

4.2.2. Polymer synthesis and development

The development of polymer can be divided in three main steps and all the polymers synthesised until achievement of the optimal composition can be classified in three main categories. The composition and synthesis of first category of polymers is based on the work of Dr Piletska and colleagues (Piletska *et al.*, 2001), who synthesised a reactive polymer for covalent immobilisation and monitoring of primary amines. The difference between new polymers and previously synthesised polymers is that whereas the polymers produced by Dr Pileska and co-workers were synthesised in

bulk form, in this work polymers needed to be immobilised on a gold sensor surface. After immobilisation the thioacetals groups contained in the polymer need to be still available for biomolecules immobilisation. The polymers mixture composition was optimised by varying the amount of allylthiol (AT) and initiator (AIBN). The composition of all the polymers synthesised initially is reported on Table 4.1. The presence of AT provides –SH groups, some of which can form thioacetal moieties after reaction with aldehyde groups, and some others permits the self-assembling of the polymer on the gold surface. The polymers also contain o-phthalic aldehyde (OPA), for creating thioacetals and fluorescent isoindole after reaction with primary amines. 2- hydroxyethyl methacrylate monomer (HEM) was added for improving water compatibility and consequently the hydrophilicity of the polymer. The synthesis was performed by overnight thermal polymerisation at 80 ^{0}C.

Table 4.1. *Composition of first group of polymers*

	Pol. 1	**Pol. 2**	**Pol. 3**	**Pol. 4**	**Pol. 5**	**Pol. 6**	**Pol. 7**	**Pol. 8**
OPA(mg)	67	67	67	67	67	67	-	67
AT(mg)	74	74	74	74	74	37	74	74
HEMA(mg)	650	650	650	650	650	650	650	650
ACN(mg)	500	500	500	500	500	500	500	500
AIBN (mg)	100	50	30	13	10	30	30	50
TEA (µl)	3	3	3	3	3	3	3	3

Pre-polymerisation mixtures were thoroughly purged with nitrogen. After polymerisation the polymers were washed three times by dissolving them in 2 ml of methanol. This was followed by precipitation obtained by adding 15 ml of ACN. Polymers were collected by centrifugation at 2500 rpm for 15 minutes.

Due to inadequate response on SPR testing it was necessary to synthesise polymers containing higher amount of thiol groups (second category of polymers). This was achieved by adding N,N Bis (acryloyl) cystamine (BAC). .The structure of BAC is illustrated below (Fig 4.2).

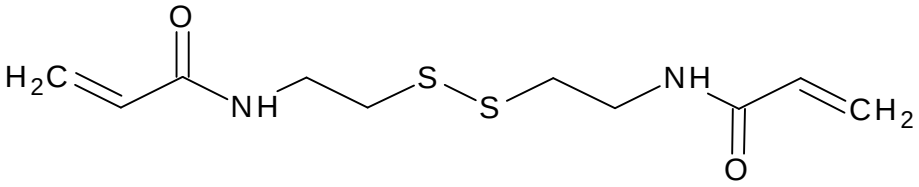


Fig. 4.2. *The structure of BAC. As it is shown BAC is a symmetric molecule and an excellent polymerisable compound because of conjugation of double bond and carbonyl bond. It can be used as a reversible cross-linker.*

The composition of polymers synthesised in this second group is illustrated in

Table 4.2.

Table 4.2. *The composition of second group of synthesised polymers.*

	Pol. 9	***Pol. 10***	***Pol. 11***	***Pol. 12***	***Pol. 13***	***Pol. 14***	***Pol. 15***	***Pol. 16***	***Pol. 17***	***Pol. 18***
AT (% w/w)	---	10	---	10	10	15	15	20	---	10
MMA(mg)	1300	1300	1300	650	250	250	250	250	250	250
DMF (g)	10	10	10	2.5	0.50	0.65	0.70	0.70	0.50	1.00
BAC (% w/w)	5	5	10	10	20	20	30	30	30	40

The above polymers were prepared by mixing the compounds and dissolving them in DMF. The monomer mixture was thoroughly purged with nitrogen, and after addition of AIBN (3% w/w) thermal polymerisation was carried out overnight at 80^{o}C. TEA (40 μl) was present in polymer mixture only when polymer contained also AT and OPA. OPA was added in polymer composition at 1:2 mol ratio of AT (Piletska *et al.*, 2001). Afterwards polymers were decrosslinked (reduction of disulphide bond of BAC) by adding DTT (Ruegg and Rudinger, 1977), at 10:1 mol ratio of BAC (Aliyar *et al.*, 2005) and 50 μl of TEA in DMF under stirring. Pol. 10 was tested with and

without decrosslinking. The time necessary for the decross-linking procedure was dependent on polymer. For polymer containing allyl thiol 2-3 hours were enough for decross-linking. However for polymers containing only BAC it was necessary to leave them overnight to obtain a successful decross-linking. The decrosslinked polymers were kept in inert atmosphere in order to avoid reoxidation of thiol groups. The polymers were washed three times with 30 ml of water according to their quantity and amount of DMF used in decrosslinking procedure. The amount of DMF was kept as low as possible in order to achieve an easier precipitation in water. Some drops of 1 M HCl were added before centrifugation for 15 min at 2500 rpm, in order to separate the precipitated polymer from the solvent.

Due to poor ability of this second group of polymers to immobilise biomolecules (Section 4.3.1.), new polymers were synthesised with an improved composition resulting on a 3-D structure.

TRIM and EGDMA were added to polymers composition and a third group of polymers (pol.19 and pol.20) with a 3-D structure were synthesised. The composition of these polymers is reported in Table 4.3.

Table 4.3. *The composition of 3-D polymers.*

Chemical	***Pol. 19 (g)***	***Pol. 20 (g)***	***Function***
2-HEM	0.5 g	0.2 g	reduce unspecific protein binding
TRIM	0.13 g	0.5 g	trifunctional cross-linker
EGDMA	0.06 g	0.06 g	cross-linker
BAC	0.03 g	0.03 g	disulphide-containing, cross-linker
MMA	0.06 g	0.06 g	facilitate polymer purification
OPA	0.1 g	0.1 g	protein binding
AT	0.16 g	0.16 g	protein binding
TEA	40 µl	40 µl	Create basic pH
BDMB	0.18 g	0.18 g	initiator
DMF	5 ml	5 ml	solvent

The polymers were synthesised by mixing the above compounds and adding TEA in order to create basic pH, which is required for thioacetal formation. The monomer mixtures were purged with nitrogen for two minutes to remove oxygen and placed

under a high intensity UV source (0.157 W/cm^2) Aprint 100 CVI (Dr. Hönle) for 20 minutes to polymerise. The synthesised polymers were precipitated from DMF by adding 2 ml of water and washed several times with methanol.

Based on pol. 20 and after a further optimisation of the composition, final polymer which was applied in the rest of the thesis, was synthesised by mixing together: 2.0 mmol (260 mg) of 2-HEM, 0.3 mmol (60 mg) of EGDMA, 1.5 mmol (507 mg) of TRIM, 1.0 mmol (134 mg) of OPA, 2.0 mmol (150 mg) of AT, 0.1 mmol (26 mg) of BAC, 0.5 mmol (180 mg) of BDMB (initiator) and DMF (5 mL) as solvent. A small amount of TEA (40 μl) was added to the monomer mixture and it was thoroughly purged with argon for 5 minutes. The polymerisation and cleaning was performed in the same way as described for polymers 19 and 20.

4.2.3. Evaluation of polymer reactivity and specificity

The assessment of the polymer reactivity was carried out by measuring the fluorescence produced by the isoindole deriving from the reaction of the thioacetal group of the polymers with primary amine groups. For the experiments 20 mg of the synthesised polymer were suspended in 3 ml of the following buffers: 0.1 M sodium phosphate buffer, pH 8.0; 0.01 M of phosphate buffered saline (PBS), pH 7.4; 0.1 M acetate buffer, pH 5.0 and pH 4.5. The emission of the suspension was measured between 400 and 460 nm in a 3 cm^3 quartz cuvette using a RF-5301 PC spectrofluorophotometer (Shidmatzu, Japan) with 370 nm as excitation wavelength. 7.5 μl NH_4OH hydroxide were added to the polymer suspension and the emission recorded after 2 minute incubation. The fluorescence maximum was recorded at 425-435 nm.

The specificity of the reaction between polymer and primary amino groups was evaluated by synthesising a polymer without OPA and by measuring both its fluorescence after reaction with ammonium hydroxide and its ability to bind proteins once immobilised on SPR sensor chips.

4.2.4. Polymer characterisation

i) Thiol groups detection and determination

During polymer development the second class of polymers were decrosslinked by reducing the disulphide bond of BAC by addition of DTT. The amount of free thiol in some of the synthesised polymers was determined by applying Ellman's reaction. Ellman's reaction is an established method to determine the presence and the amount of thiol groups in a variety of substances. When the Ellman's reagent is mixed with a compound containing thiol groups, a disulphide bond is formed and a thiolate ion is released, see scheme shown in the Fig. 4.3. This thiolate ion is coloured and can be quantified using a UV-visible spectrophotometer at 412 nm.

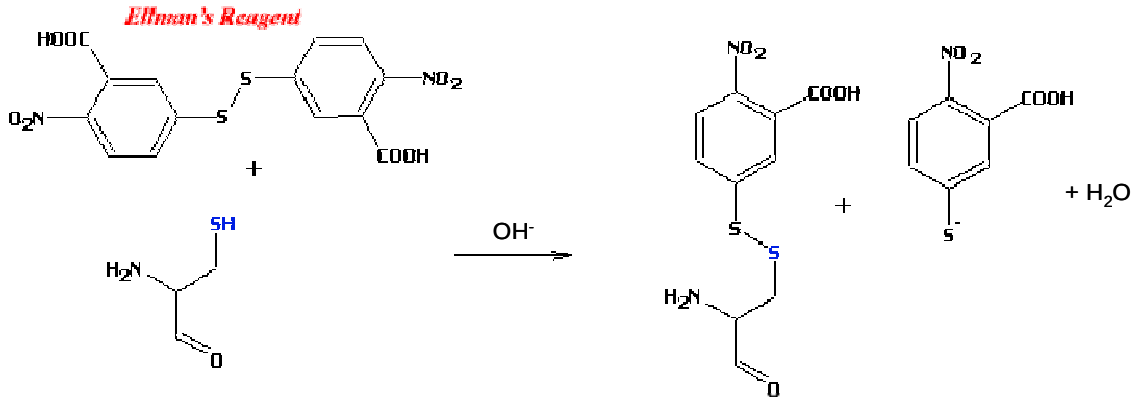


Fig. 4.3. *Schematic representation of Ellman's reaction (http://www.proteinchemist.com/chemistry/ellmans.html)*

A description of protocol of the Ellman's reaction is used here follows. In order to obtain a calibration curve the standards were prepared using DTT as source of thiol groups. The maximum absorbance was measured at 412 nm. The measured samples

were prepared by adding 40 μl of either standard DTT solutions in range of 0.00005-0.0025 M, or polymer solutions (2 mg ml^{-1}) to 100 μl Ellman's reagent dissolved in 500 μl of ethanol and mixed with 400 μl of phosphate buffer (0.1 M pH 8.0). The control sample contained 540 μl of ethanol solution instead of 500 μl, because of the absence of any thiol. The mass of the polymer in solution was determined by drying 1 ml of polymer solution and weighting the solid remained.

ii) Determination of polymer's size – GPC Chromatography

In order to determine the size of synthesised polymers, Gel Permeation Chromatography (GPC) was applied. GPC is a separation method for the determination of molecular weight averages (Mn) and molecular weight distributions (PDI = Mw/Mn) of polymers. GPC is considered an analytical technique of high importance and its use is well documented in the literature although it is still not widely used for the fractionation of polymer samples on a preparative scale. In GPC, the stationary phase is a swollen gel made by polymerising and cross-linking styrene in the presence of a diluent, which is a non solvent for the styrene polymer. The polymer that needs to be analysed is injected into the column and then is eluted with a mobile phase. The polymer molecules diffuse through the gel at a rate depending on their molecular size rate. Separation takes place according to molecular size. High molar masses are eluted firstly and then small molecules (low molar masses) follow. Thus, the method can be used for analytical work, determination of oligomer content and purification of polymer samples. An important extension of GPC in polymer synthesis is the opportunity to purify polymers by removing low molecular weight impurities, e.g., residual initiator or additives. These low molecular weight

components are eluted after the main fraction of the polymer and can be easily removed.

In this work GPC was used for the determination of an approximate molecular weight Mw of the polymers synthesised. Therefore polymers were dissolved in THF and 20 μl of 1 mg ml^{-1} solution were injected for analysis. The experiments were carried out using an Agilent 1100 SERIES HPLC system. HPLC analysis was performed using THF as mobile phase at a flow-rate of 1.0 ml min^{-1} and with UV detection at 268 nm. The column was Phenogel with 5 μm particles size and dimensions 300 × 780 mm (Phenomenex®). The column was calibrated with polystyrene standards of range of 13000-106000 Da purchased from Phenomenex and polystyrene standards of range of 500-10000 Da purchased from Fluka. The size of polymer particles was calculated from calibration curve according to the retention time.

4.2.5. Polymer testing

Biacore 3000 (Sweden), Au-coated chips (SIA Kit Au), CM5 and CM3 chips, purchased from Biacore (Sweden), were used in this work. All the experiments were performed at room temperature (25° C).

4.2.6. Treatment of gold chips – gold surface modification

Sensor chips, SIA Kit Au (Biacore, Sweden) were used in order to assess the ability of polymer-coated surfaces to bind proteins. SIA Kit Au chips were cleaned for 3 minutes using oxygen plasma at 40 W in a plasma chamber (Emitech, UK). Polymer was self-assembled onto SIA Kit Au by immersing chips in 5 ml acetone/ethanol 50/50 (v/v) containing 10 mg ml^{-1} of polymer solution for 24 h. The polymer-coated gold chips were rinsed thoroughly with acetone/ethanol, dried with nitrogen and

assembled onto the holder. In case of AT-cut quartz crystals cleaning for 5 minutes at 50 W was necessary to obtain clean surface and the polymer attachment was made in the same way as for SIA Kit Au chips.

4.2.7. Gold surface characterisation

Static contact angle measurements were made using a CCD camera supplied by Spectra Source Equipment model MCD400S (USA) with the software provided. The hydrophobicity/hydrophylicity of modified gold chips was determined by measuring the interfacial tension of a drop of water on polymer-coated surface. The thickness of layers produced by the applied polymer was measured by ellipsometry and by atomic force microscopy (AFM). For the ellipsometry, the extinction coefficient of bare gold chips and the thickness values for polymer coated sensors (seven sensor chips, three measurements per chip) were obtained using a SE 400 Ellipsometer (Mi-Net Technologies Ltd., UK). The roughness of the layers produced by the applied polymer was also measured using atomic force microscopy performed in contact mode using a PicoScan SPM from Molecular Imaging (USA). The deposition of second group of polymers was measured by coating AT-cut quartz crystals (14 mm diameter, ICM, USA) resonating at 10 MHz with Cr/Au electrodes (6 mm diameter). The experiments were performed in a batch-cell with the sensor positioned in the cell. This batch-cell was directly connected to the Libra α Nanobalance Measuring System (Technobiochip, Italy) recording the frequency. The Libra nanobalance was interfaced with a PC for the data acquisition and processing. The system was driven by LIBRA 3.1 software running under MS-Windows^{TM}. The frequency of crystal was measured before and after polymer deposition on the surface.

4.2.8. Protein immobilisation on polymer-coated and carboxydextran surfaces

The protein immobilisation on sensor surfaces was monitored by Biacore 3000, which is a surface plasmon resonance (SPR) based instrument with a continuous flow system and four flow channels. The change in Biacore response units (RU) is directly proportional to the change of surface mass; 1 RU is approximately equivalent to 1 pg mm^{-2}. The biomolecules used for studying the performance of the polymer-modified surface, were BSA, calf Thymus DNA (for the first and second class of synthesised polymers) the enzymes trypsin, carbonic anhydrase and pepsin, sheep polyclonal anti-microcystin-LR Ab, anti-sheep IgG Ab and mouse monoclonal anti-PSA Ab. Mainly BSA but also Calf Thymus DNA were used as model proteins to assess the polymers binding capacity for biomolecules immobilisation. CM3 and CM5, which are Biacore carboxymethylated dextran chips used for biomolecule immobilisation, were tested for comparison. In this case, CM3 and CM5 were activated with EDC/NHS chemistry (Fagerstam et al., 1992), while polymer-coated surface did not require any activation. The activation of CM3 and CM5 was performed by injecting 30 µl of 0.2 M EDC/0.05 M NHS. Typically protein immobilisation was carried out on polymer-modified surfaces by injecting 100 µl of 100 µg ml^{-1} of protein solution in 0.01M phosphate buffered saline, pH 7.4 with a flow rate 15 ml min^{-1}. In case of pH study for protein immobilisation on the best performing polymer protein solution was prepared in 0.1 M acetate buffer pH 4.5, 0.1 M acetate buffer pH 5.0 and 0.1 M phosphate buffer pH 8.0. Immobilisation of proteins onto CM3 and CM5 was performed in 0.1 M Na-acetate buffer, pH 5.0. The stability of biomolecules immobilisation on polymer-modified surfaces was tested by passing 5 µl of regeneration solution: 0.1%, SDS (sodium dodecyl sulphate) at a flow rate of 35 µl min^{-1}. For the immobilisation of antibodies (anti-microcystin-LR and anti-PSA Ab)

100 µl (two injections of 50 µl) of antibodies solution (1/1000 dilution for anti-microcystin-LR and 2 µg ml^{-1} of anti-PSA) were injected in PBS with flow rate of 15 µl min^{-1}. After covalent coupling of the antibodies, remaining thioacetal groups were deactivated by injecting 50 µl of 0.2 M Ethanolamine with a flow rate 10 µl min^{-1}. The assessment of the reactivity of the immobilised antibodies was carried out by injecting corresponding antigens such as anti sheep IgG and microcystin-LR for Sheep anti microcystin–LR and PSA for anti-PSA . The antigens were diluted in PBS buffer and injected for 3-5 minutes, with a flow rate of 10 µl min^{-1}. In all experiments a reference channel containing polymer blocked with BSA and ETA was used for assessment of binding specificity. The surface was regenerated by injecting 5-10 µl of 10 mM HCl at a flow rate of 30-35 µl min^{-1}. The detection limit (LOD) was calculated as the minimum analyte concentration, which produced a signal at least three times higher than the signal of analyte recorded on the reference channel which is consider as the background noise.

4.3. Results and Discussion

4.3.1. Polymer Synthesis and Development

The composition of the first group of polymers synthesised, see Table 4.1, is based on the work of Piletska *et al.* (2001). Thus they contain AT, which is a polymerisable compound containing thiol groups and OPA for thioacetal formation in a basic environment, created with the addition of TEA. 2- hydroxyethyl methacrylate (2-HEM) (monomer) is added for improving water compatibility. When the first group of polymers were tested using a fluorescence spectrophotometer the highest amount of fluorescence was generally recorded 50 minutes after addition of NH_4OH and the

fluorescent profiles recorded for pol. 5, which showed the highest signal, is depicted in Fig. 4.4.

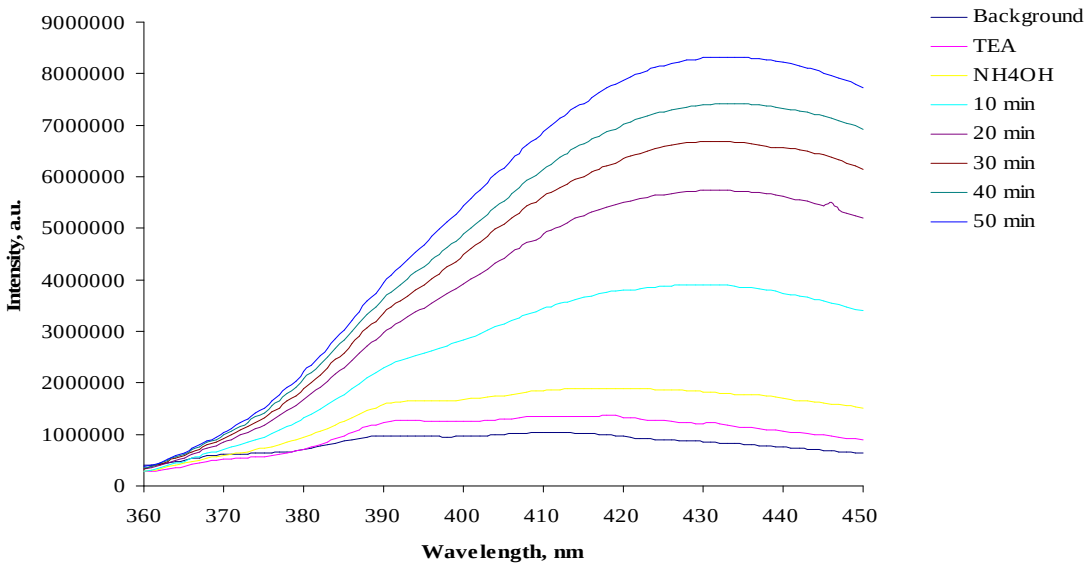


Fig. 4.4. *The relation between time of reaction with NH_4OH and the resulted fluorescence intensity of pol. 5.*

According to the measurements carried out with the Shidmatzu RF-5301 PC fluorescence spectrophotometer, the fluorescence values recorded in arbitrary units varied from 22.6 for pol.1 to 58.4 for pol. 5. An inverse correlation between the amount of the initiator used for the polymer synthesis and the recorded fluorescent was present. This may be related with the rigidity of polymers formed and the amount of availability of active groups for reaction with primary amines. The polymer with less initiator in polymer mixture performed better than the other polymers. In fact low amount of initiator produces polymer with more flexible structure and therefore with a higher availability of thioacetal groups. Thus pol. 5 was applied for further testing. Its deposition on gold surface was measured with QCM testing as it was described in material and methods (see 4.2.7, 4.2.8) and the difference of signal before and after polymer deposition was 94 ± 37 Hz for set of three measurements which confirmed

the deposition of polymer on the gold surface. The high STD of QCM signal could be due to the surface morphology, fluctuations in room temperature, which can affect heavily QCM signals and instrument stability.

SPR testing with Calf Thymus DNA and BSA showed a poor ability of pol. 5 to immobilise proteins on sensor surface. In fact the remaining DNA and BSA, after a SDS washing, produced sensor signals of 294.3 ± 73.6 RU and 83.1 ± 16.6 RU respectively. The low level of immobilisation could be due either to the presence of a limited amount of binding sites in the polymer or to a low availability of thioacetal groups for interaction with primary amines of biomolecules.

In the attempt to increase the amount and availability of thioacetal groups in the polymer, a new family of polymers was synthesised with the addition of BAC, as a source of disulphide groups. In fact addition of BAC in polymer composition should, increase the amount sulphur groups in resulting polymers. Increment of sulphur groups could have also be achieved by increasing the amount of AT in polymer mixture, with the risk, however of inhibiting the polymerisation reaction, due to the formation of radicals which are stabilised through conjugation.

Firstly, the polymer reactivity was assessed by fluorimetry after reduction of disulphide bonds by applying DTT as a reduction reagent. There was a significant improvement in polymers performance, since the recorded fluorescence was 182.3 ± 17.3 to 312.9 ± 43.8 units for polymers containing AT/OPA (pol.10, pol. 12, pol. 13, pol. 14, pol.15, pol. 16, pol. 18) and 393.7 ± 50.0 to 836.7 ± 159.8 units for polymers without AT/OPA (pol. 9, pol. 11, pol. 17). This was expected, since for these latter polymers, containing only BAC instead of AT, OPA was added in the solution for fluorescence testing and consequently higher amount of thioacetals were formed and they were available for the reaction with NH_4OH comparing to polymers where the

only available thioacetals were formed during polymer synthesis. In fact during polymerisation there is possibility that some thiol groups will do not form thioacetals by reaction with OPA present in solution since thiols groups are very susceptible to oxidation and other reactions during polymerisation. Among the polymers containing AT, those with the highest amount of AT in their composition showed the highest fluorescence as it was expected since they contained higher amount of thioacetals (pol.16). Also the polymer containing higher amount of BAC (pol. 17) exhibited high fluorescence performance among the polymers containing only BAC. This was expected due to the presence of higher amount of thiols and eventually thioacetal groups after reaction with OPA. Pol.10, which was also tested when not decrosslinked, did not show significant difference in fluorescence performance (182.3 ± 17.3 units) to when decrosslinked pol.10 (212.8 ± 20.2 units). The slightly higher fluorescence of decrosslinked pol.10 and higher STD could be due to the presence of DTT residues, remained after polymer washing.

The fluorescence reactivity of polymers without AT/OPA is an indication of successful reduction of disulphide bonds since disulphide groups would not show any fluorescence after reaction with OPA and primary amines. The presence of thiol groups in polymers was confirmed with Ellman's test since increase in BAC content in polymer synthesis results in an increase of the amount of –SH groups in polymer after decrosslinking. As it is will shown later decrosslinking was not important for polymer performance in SPR. In fact attachment of polymer on the surface could take place through disulphide groups with difference only in the kinetics of the reaction, which is slower for disulphide than thiol groups (Biebuyck *et al*., 1994).

The deposition of pol. 10 (decrosslinked and not decrosslinked), pol. 11 and pol. 16 was also recorded on the gold surface of QCM electrodes (see sections 4.2.7, 4.2.8).

The average shifts of the signal calculated by using two experiments: 89.5 Hz for pol. 10 (decrosslinked), 212.0 Hz for pol. 10 (no decrosslinked), 600.8 Hz for pol. 11, 425.5 Hz for pol. 14 and 192.3 Hz for pol. 16. According to QCM results, the polymers seem to contain reasonable amounts of available –SH or –S-S groups for attachment onto gold surfaces. The shifts of QCM signals were proportional to the amount of polymer immobilised onto the surface but also correlated with the size of immobilised molecules. It was clear that the presence of AT in polymer mixture resulted in lower QCM signals and this could be possibly due to the smaller size of the polymers immobilised. Also the higher signal observed on QCM for non decrosslinked polymer showed that the decrosslinking is not necessary for polymer deposition onto gold surfaces and the lower signal recorded for decrosslinked polymer could be due to a smaller size of polymer fractions. The size of the synthesised polymers after decrosslinking varied as it was demonstrated by using Gel Permeation Chromatography (GPC). According to retention times, the polymers are consisting of polymer fractions ranging from 4 kDa to 100 kDa.

After the encouraging fluorescence and QCM results pol. 10, pol. 11, pol. 14 and pol. 16 were applied on SPR for Calf Thymus DNA and BSA immobilisation. Surprisingly no significant improvement on protein binding (less than 20% increment of biomolecules immobilisation comparing to polymers 1-8) was recorded and the very low protein capacity did not allow further SPR experiments.

Taking all the results into consideration and especially the disparity between the positive fluorescence data and the poor SPR performance, it became clear that the structure of polymer had to be improved. In order to increase the availability of thioacetal groups for protein immobilisation once polymer is attached on gold surfaces, a tri-functional crosslinker (TRIM) was added in polymer composition. The

composition of polymer was optimised in order to obtain polymers soluble in ethanol, which facilitates immobilisation after plasma cleaning due to the ability of this solvent to reduce gold oxides, and relatively insoluble in water and methanol, solvents used for precipitation and washing correspondingly. These solubility characteristics were obtained with the addition of high amount of TRIM and BAC. As explained earlier, high amount of AT in polymer mixture was, on the other end, avoided as this could inhibit polymerisation producing smaller polymer fractions, which could result in pinholes with uncovered gold surfaces and consequently more non specific binding. Pol. 20, which contained higher amount of TRIM in its composition, showed higher binding capacity on SPR comparing to pol. 19. The recorded SPR signal for BSA immobilisation on polymers 19 and 20 was 479.7 ± 25.1 RU and 1087.2 ± 66.4 RU correspondingly. This is another indication showing that the low binding capacity observed for the previous groups of polymers was directly related to their structure. Therefore the synthesis of the polymer applied finally in this work was based on that of pol. 20 with further improvements in the composition.

4.3.2. Properties of the developed 3-D polymer

o-Phthaldialdehyde and allylthiol in the presence of triethylamine react to create thioacetal groups, which can in turn react with primary amino groups with formation of a fluorescent isoindole. As mentioned previously, BAC was included in the polymer composition to supply disulphide groups suitable for covalent attachment of the polymer on gold surfaces. 2-HEM was included in polymer composition to increase the hydrophilicity of the matrix, as this is associated with reduction of non-specific proteins adsorption. EGDMA is a bifunctional cross-linker and TRIM is a trifunctional cross-linker, which leads to a 3D polymer network and therefore to a larger surface area and higher availability of protein binding sites. In the absence of

TRIM and with only EGDMA as crosslinker, the polymer capacity for proteins was up to 10 times lower. The main advantage of the 3D polymer lies in the opportunity to avoid binding site hindrance, which could be present in high density and flat antibody layers. Together with incorrect antibody orientation, this could lead to a high percentage of unavailable binding sites. The tri-dimensional network leads to a random immobilisation of antibodies far from the sensor surface, but still in close proximity to allow the detection of antigen binding (see Fig. 4.1). When the reactivity of the the 3-D polymer was assessed in solution at different pH values (by measuring the fluorescence of the formed isoindole group) a greater response was recorded in basic pHs. Significantly lower fluorescence was observed for the polymer suspensions in acidic medium. The fluorescence intensity, which was recorded 2 minutes after addition of NH_4OH 6 M, was 615.7 ± 35.7 units at pH 8.0; 512.3 ± 41.0 units at pH 7.4; 64.4 ± 14.8 units at pH 5.0 and 55.2 ± 18.7 units for pH 4.5. The experiments were performed in triplicate. The presence of fluorescence is a proof of the isoindole formation and therefore of the existence of thioacetal groups. The results also demonstrate the suitability of the polymer to perform protein immobilisation at physiological pH, which can be advantageous to avoid protein denaturation. The average molecular weight of the polymer, as determined by GPC was 110 kDa (polystyrene equivalent).

The specificity of protein immobilisation on the 3-D polymer by amino coupling resulting isoindole formation was demonstrated by recording the fluorescence and the performance on SPR of a ‘control polymer’, which was synthesised with the absence of OPA. No fluorescence was observed during fluorimetric application.

4.3.3. Characterisation of polymer-coated sensor surfaces

The 3-D polymer was attached to sensor gold surfaces by immersing SPR chips in a polymer solution as explained in Section 4.2.6. The characterisation of the obtained polymer layer included contact angle measurement ellipsometry and atomic force microscopy. Contact angle measurements showed a moderately hydrophobic gold surface after modification with the polymer with an angle of $67.2^{o} \pm 6.0^{o}$ (a very hydrophobic surface would have a value greater than 90^{o}). The average thickness of the polymer coating was measured as 5.3 ± 1.1 nm using an ellipsometer. The layer is relatively thin if compared to Biacore chips CM3 and CM5 (commercially available SPR chips for biomolecules immobilisation), where, after exposure to aqueous solutions the carboxydextran layer are 30 nm and 100 nm correspondingly. Thicker layers were obtained by leaving the chips for longer time in the polymer solution (up to one week). This resulted not only in an increased capacity for protein, but also in an increased non-specific binding, so a thinner layer was considered optimal for further sensor work. The small deviation in the ellipsometry value shows that the polymer coating is homogeneous. For further characterisation of the 3-D polymer layer on SIA Kit Au sensor surface, AFM measurements were carried out in contact mode [PicoScan SPM from Molecular Imaging (USA)]. As it can be seen from Fig. 4.5 a and b, there is a change in the surface texture after the immobilisation of the polymer. A decrease in surface roughness was observed in presence of polymer (from 0.80 nm to 0.66 nm).

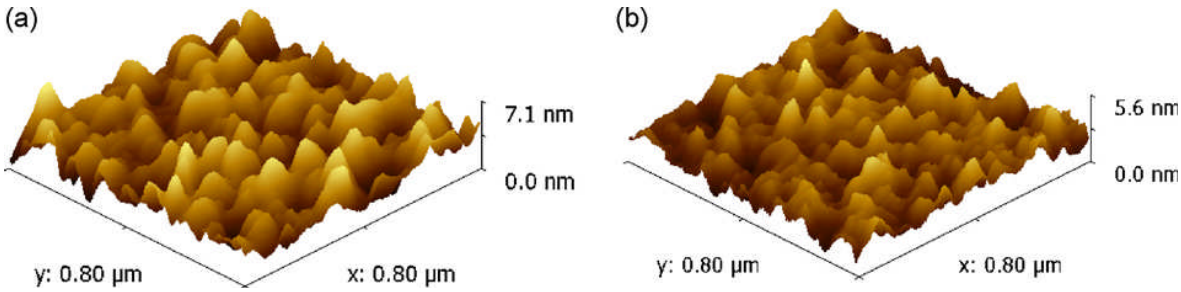


Fig. 4.5. *AFM topographies of (a) cleaned gold substrate and (b) polymer-coated surface with respective roughness of 0.80 nm and 0.66 nm.*

4.3.4. Evaluation of Polymer performance by SPR

The evaluation of the ability of the 3-D polymer to bind proteins after attachment on a sensor surface was performed using Biacore 3000. BSA was employed as model biomolecule and a protocol for protein immobilisation on polymer was optimised. The attachment of BSA to the polymer-coated surface showed a satisfactory immobilisation level, which produced a Biacore signal of 1595.9 ± 51.2 RU with the experiments repeated 12 times. The immobilised BSA was washed with 10 µl 0.1% SDS solution (flow rate 35 µl min^{-1}) in order to assess the affinity of the immobilisation. The material was relatively stable on the surface, and less than 5% of the immobilised BSA was removed from the sensor surface by the washing step. The BSA removed during the first washing step was most likely the one loosely adsorbed on the surface. The remaining BSA (covalently immobilised) was stable and no further material losses were detected. The specificity of protein immobilisation on the polymer surface was assessed by recording the performance of the control polymer (without OPA) for BSA immobilisation on SPR. The amount of immobilised BSA on this polymer after washing with SDS 0.1% was 315.1 ± 34.8 RU, which is significantly lower than the signal obtained immobilising BSA on polymer containing OPA. The BSA immobilisation recorded on the surface of the control polymer could

be due mainly to hydrophobic interactions, hydrogen bonds or holes on the polymer layer.

4.3.5. Effect of pH on BSA immobilisation

It is well known that protein immobilisation is highly dependent on pH (Branden and Tooze, 1999). In this work effect of a pH range of 4.5–8.0 (0.1 M acetate buffer pH 4.5, 0.1 M acetate buffer pH 5.0, 0.01 M PBS buffer pH 7.4 and 0.1 M phosphate buffer pH 8.0) on protein immobilisation was studied using BSA (100 μg ml^{-1}) as a model protein. The results are illustrated in Fig. 4.6.

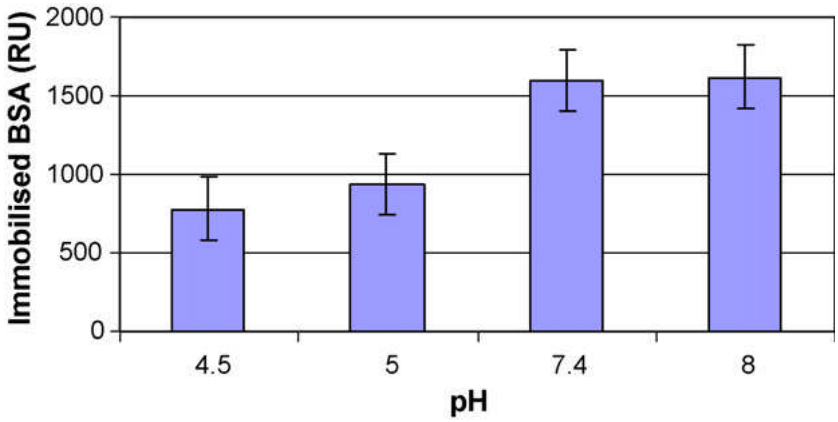


Fig. 4.6. *Influence of buffer pH on the immobilisation of BSA (100 μg ml^{-1}) onto polymer-coated surfaces. Error bars represent the STD of Biacore responses (RU) for immobilisation of BSA on polymer sensor surface in different pH. STD was calculated from a set of 3 experiments.*

The experiment was carried out in triplicate. The result is consistent with the fluorescence experiments described above, where the lowest fluorescence was recorded at pH 5.0 and 4.5. The decrement of protein binding in acidic conditions could be due to an inhibition of the isoindole formation at low pH and also to variations in protein charges induced by pH. Thus it is possible that more polar, negatively charged BSA at low pH would have lower affinity to relatively

hydrophobic polymer surface. The highest immobilisation was achieved with PBS buffer pH 7.4 and 8.0. We believe that pH 7.4 would be ideal for protein immobilisation since it resembles physiological conditions.

4.3.6. Study of the bioreactivity of the immobilised antibodies

The maintenance of biorecognition activity of the ligands after immobilisation and minimisation of non-specific interactions between the sample and the recognition element are crucial for biosensor development. Thus, polyclonal anti-microcystin-LR Ab and monoclonal anti-PSA Ab were immobilised onto 3-D polymer coated surfaces in order to study their ability to interact with their corresponding antigens microcystin-LR and PSA. Initially the performance of sheep microcystin–LR Ab was assessed by using anti-sheep IgG as antigen. The antibody was immobilised on the surface by using dilutions of 1/100 and 1/1000. The results of the immunoreactions between this immobilised antibody and both anti- sheep IgG and microcystin– LR are illustrated in Table 4.4.

Table 4.4. *Immobilisation of Sheep IgG and binding of anti-Sheep IgG and microcystin-LR on biosensor surface.*

	Concentration/ Dilution	***n****	***ΔRU(Biacore ResponseUnits)***
Sheep IgG Antibodies	1/100	3	4618.7 ± 277.1
	1/1000	12	2350.1 ± 275.0
Anti-Sheep IgG Antibodies	1/100	3	1922.6 ± 378.2
	1/1000	3	1263.2 ± 21.5
Microcystin- LR	10ppb	3	49.7 ± 3.8
	50ppb	3	112.1 ± 7.2
	100ppb	3	248.4 ± 22.6

** number of experiments*

The performance of the sheep IgG anti-microcystin–LR Ab towards anti sheep IgG was satisfisfactory since the recorded signal for antigen binding was 1683.9 ± 21.5 RU and the corresponding signal on the reference channel, containing polymer blocked with BSA (Kim *et al.*, 2007), was 420.7 ± 89.6 RU for 1/1000 dilution. After subtracting the non specific response the remaining response (1263.2 RU) shows more than 50% availability of binding sites according to a 1:1 Langmuir binding. The same conclusion was also evident when, after immobilising a dilution 1/100 of anti-microcystin Ab (4618.7 ± 277.1 RU) the anti-sheep Ab (specific for sheep anti-microcystin-LR Ab) was injected in the system and signals of 2456.9 ± 378.2 RU, and 534.3 ± 66.8 RU were obtained for working and reference (with polymer blocked by BSA) channels, respectively. The relatively high amount of non specific binding could be due both to the hydrophobic character of the polymer and through non specific interactions between the antibody and immobilised BSA on reference channel. These results highlight the good availability of binding sites on the immobilised antibodies, with around 42% available, calculated considering that anti-microcystin- LR Ab and anti-sheep Ab have similar molecular weight.

Microcystin–LR testing was performed using only the 1/1000 dilution of Ab, see Table 4.4. The sensogram showing the immobilisation of anti microcystin- LR Ab and the immunoreaction with injection of one concentration of microcystin-LR is illustrated in Fig. 4.7. The figure also shows the regeneration step and the possibility to reuse the antibodies for further binding cycles. The antibodies seemed to keep their bioreactivity for three regeneration cycles by using the applied regeneration conditions. The limit of detection for microcystin-LR was 10 ng ml^{-1}. This detection limit for the toxin is relatively high in comparison to that obtained when the same antibodies were first used (Chianella *et al.*, 2002). This can be related both with the

aging of the Ab, which was stored frozen for longer than 6 years, and most likely with the differences between the detection methods used in the two works: ELISA in the past *versus* direct SPR detection of a small analyte such as microcystin-LR in the current work.

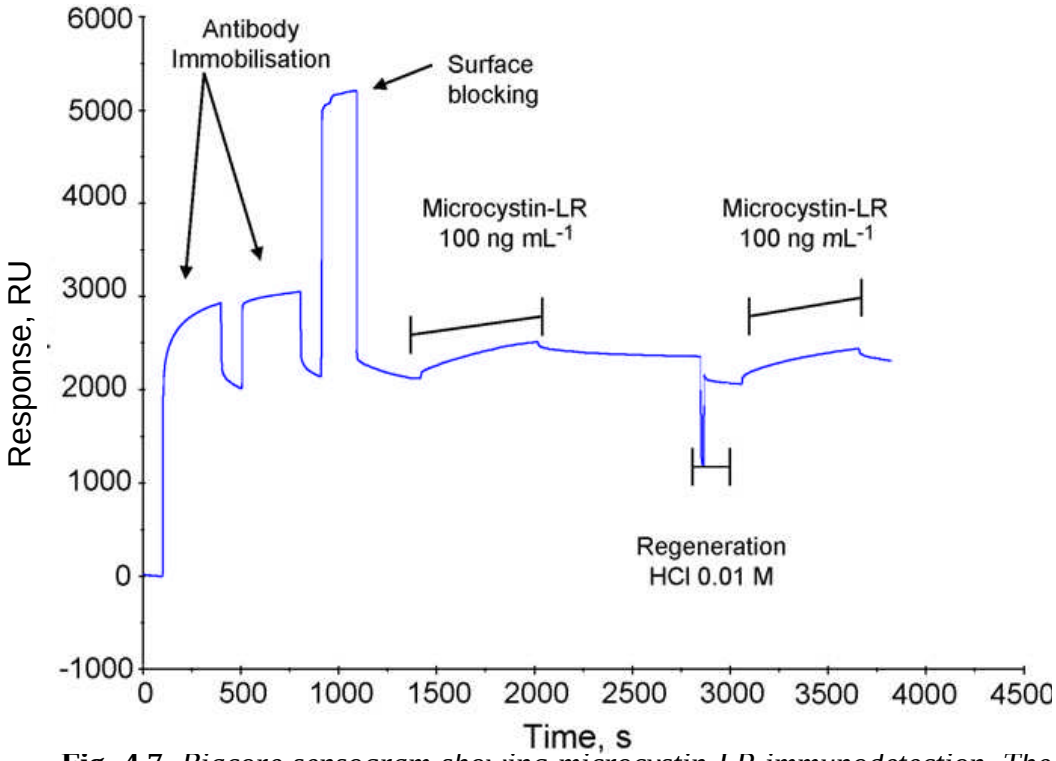


Fig. 4.7. *Biacore sensogram showing microcystin-LR immunodetection. The first two injections correspond to the 1/1000 antibody (polyclonal anti-microcystin-LR Ab) immobilisation; blocking of unreacted binding sites with 1M ETA, pH 8.5 follows. The binding of 100 ng ml^{-1} microcystin-LR then occurs by a 50 µl injection in PBS buffer, pH 7.4 with a flow rate of 5 µl min^{-1}. Regeneration was performed by injecting 10 mM HCl for 10 s. At the end, a rebinding of microcystin-LR to regenerated Ab showing the same performance as in the first cycle is reported.*

The amount of non-specific binding of analytes to the polymer was evaluated using a reference channel. In this channel the polymer layer was blocked with BSA (Kim *et al.*, 2007) by injecting 100 µl of 100 µg ml^{-1} of the protein in PBS buffer, pH 7.4, followed by ethanolamine (ETA) blocking. The binding of the highest tested

concentration of microcystin-LR (100 ng ml^{-1}) on this channel was negligible (lower than 20.0 RU).

In another set of experiments, still aimed at showing the performance of the 3-D polymer for immobilisation of biomolecules, the binding of PSA to monoclonal anti-PSA Ab attached to polymer-coated chips was studied, after optimising the immobilisation of the antibody. In fact, firstly, several concentrations of anti-PSA Ab were immobilised on to the 3-D polymer (2.0 μg ml^{-1}, 1.0 μg ml^{-1}, 0.2 μg ml^{-1}, 0.1 μg ml^{-1}, 0.05 μg ml^{-1}, 0.02 μg ml^{-1}) followed by injections of the antigen PSA to assess performance. The highest sensor responses were obtained with 2 μg ml^{-1} (1976.4 ± 181.8 RU), which also provided the lowest detection limit of PSA. This value was calculated for a set of 24 immobilisations and the low STD demonstrates a good reproducibility, which could be further improved by automation of the polymer coating procedure. The calibration curve for PSA obtained with this amount of anti-PSA Ab is shown in Fig. 4.7. The detection limit determined according to the calibration curve and to the non specific binding on the control channel (the first concentration which gives higher than 3 times the response of analyte obtained on the control channel) was determined at 0.05 ng ml^{-1}. This low limit of detection could be due to the use of monoclonal antibody (instead of polyclonal) and the easy accessibility of the binding sites resulting from the 3-D structure of the polymer, with approximately 90% binding site availability calculated for a 1:1 Langmuir binding. In fact immobilisation of anti-PSA Ab (150 KDa) produced a signal of 1976.4 ± 181.8 RU and the net signal for the highest concentration PSA (34 KDa) was 420.5 ± 26.1 RU.

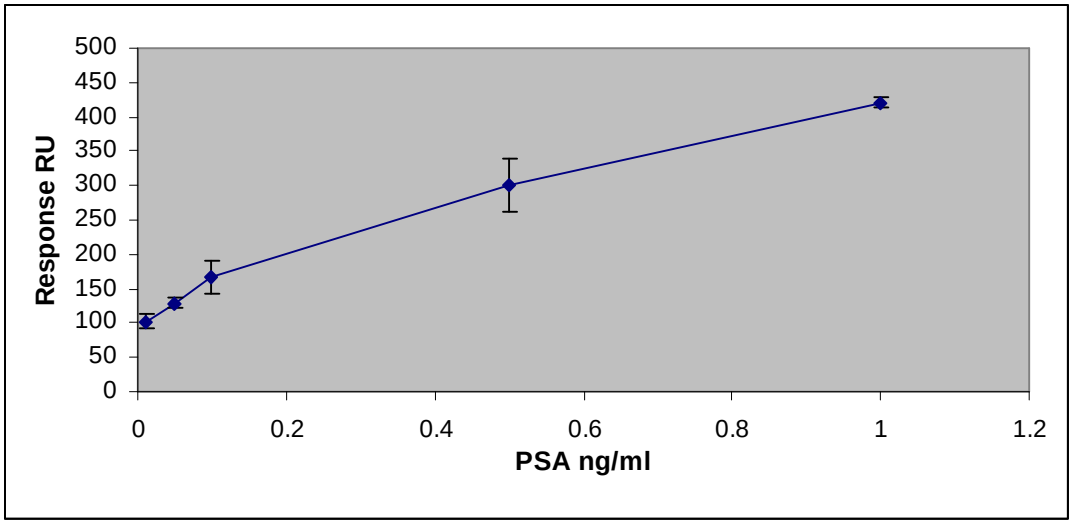


Fig. 4.8.*Calibration curve of PSA obtained with direct assay, using monoclonal anti-PSA Ab immobilised on the polymer-coated surface. Error bars represent the STD of Biacore response (RU) for each PSA concentration and it was calculated from a set of 3 measurements.*

The higher number of available binding sites when compared with the anti-microcystin/anti-(anti-microcystin) system described above might be explained with improved accessibility/diffusion of PSA due to the lower molecular weight (34 kDa for PSA and 150 kDa for IgG). The detection limit achieved in our system (0.05 ng ml^{-1}) is considered relatively low for direct assay since lower (0.027 ng ml^{-1}) LOD obtained only by enhanced SPR methods (Cao and Sim, 2007; Choi et al., 2008). This could be due to the high availability of binding sites of antibodies immobilised on 3D-polymer surface and the fact that the antibodies were not modified with biotin or other capture reagent since modification can alter their bioreactivity.

Regarding the stability of the novel matrix, polymer-coated chips stored at room temperature and exposed to air for up to 2 months did not show any deterioration in performance. A longer stability study (up to 6 months) was not possible at the time.

For comparison purposes, the monoclonal anti-PSA Ab were also immobilised onto Biacore carboxymethylated dextran CM3 chip, using the immobilisation protocol

suggested by the company. A very low antibody immobilisation was observed (301.9 RU) possibly due to the presence of sodium hydrazide in the antibody solution which can prevent preconcentration of the proteins on carboxymethylated dextan surface. The quantity of the antibody immobilised on the chip was so low that it was difficult to detect the antigen PSA even at the highest concentrations injected. This shows that the novel matrix produced in this work is far superior to CM3 commercially available chips.

The calibration curve reported in Fig. 4.8 was obtained after subtraction of the non-specific binding, which was assessed by the amount of antigen binding to the reference channel (polymer channel blocked with BSA). The non-specific binding of PSA was increasing proportionally with PSA concentration; see Table 4.5.

Table 4.5. *PSA binding in the range of 0.01 ng ml^{-1} - 1 ng ml^{-1} on sensor surface with immobilised anti PSA (mouse monoclonal). Binding to working channel, reference channel and net binding is reported.*

PSA (ng ml^{-1})	***PSA binding on working channel [anti PSA] (RU)***	***PSA binding on reference channel [BSA]***	***Net PSA binding[Working channel-Reference channel] (RU)***
0.01	112.4 ± 9.9	10.3 ± 1.1	102.1
0.05	155.5 ± 7.0	26.4 ± 4.1	129.1
0.1	232.4 ± 23.0	65.1 ± 6.8	167.3
0.5	440.4 ± 39.7	140.0 ± 25.1	300.4
1	617.6 ± 8.1	197.1 ± 41.0	420.5

* *Standard deviation was calculated from set of 3 experiments*

This high value of non specific binding could be due to a combination of factors. Firstly a channel blocked with BSA might not be good enough in assessing the non specific binding of a channel containing antibody. In following development of the work (Chapter 5) a different type of Ab was immobilised in reference channel for

assessment of non specific binding. Secondly, the hydrophobicity of the polymer could also contribute to the non specific protein adsorption through hydrophobic interactions.

4.3.7. Influence of pI on protein immobilisation

A challenging aspect during the development of sensors is the immobilisation of proteins with different isoelectric points (p*I*). Therefore it was important to study the possibility of immobilising very different proteins on the novel 3-D polymer. For these experiments three enzymes (pepsin, carbonic anhydrase and trypsin) with p*I* ranging form 1 to 10 were tested. The results were then compared with those obtained with the same enzymes immobilised onto commercial Biacore chips CM3 and CM5. Both CM3 and CM5 are carboxymethylated dextran chips and biomolecules can be covalently attached to their surfaces by a peptide bond formation after activation of the carboxyl groups. The main difference between the two chips is the length of the carboxydextran matrix. The thickness of the dextran layer, once in contact with water, is 100 nm and 30 nm for CM5 and CM3, respectively, whereas the thickness of our polymer layer in a dry state is around 5 nm as measured by ellipsometry. Therefore pepsin (p*I* = 1.0), carbonic anhydrase (p*I* = 6.5), trypsin (p*I* = 10) and BSA (p*I* = 4.5–5.5) were immobilised onto polymer coated surface, CM3 and CM5. The results of the immobilisations are reported in Table 4.6. All the experiments were performed in triplicate.

Table 4.6. *The immobilisation values of 5 proteins on polymer goated SIA Kit (Au), CM3 and CM5. The results obtained by using Biacore 3000.*

Protein	***pI***	***Polymer coated chip (RU)***	***CM3 (RU)***	***CM5 (RU)***
Pepsin	1	1164.7 ± 46.6	56.5 ± 12.1	39.0 ± 9.0
BSA	4.5-5.5	1595.9 ± 51.2	1232.6 ± 229.3	12030.4 ± 830.1
Carbonic Anhydrase	6.5	1780.3 ± 60.0	4450.6 ± 31.2	6845.1 ± 143.7
Trypsin	10	1716.4 ± 39.5	499.9 ± 61.0	4754.6 ± 351.8

As suggested by Biacore the immobilisation buffer used for CM3 and CM5 was 0.1 M acetate buffer, pH 5.0. At this pH a high negatively charged carboxydextran matrix is generated. Thus this charged matrix binds preferentially to positively charged proteins (proteins with p*I* higher than 5.0). Consequently all the proteins with a p*I* lower than 5.0 and therefore with a negative charge are not able to easily approach the dextran layer. In addition at pH 5.0 some proteins might start denaturating. Hence the possibility of using a buffer with pH 7.4, which is very close to physiological conditions, for the immobilisation of ligands onto the polymer is a significant advantage. In fact Table 4.6 shows that successful immobilisation of pepsin (p*I* = 1.0) was achieved only on the polymer, whereas as expected practically no attachment was observed on CM3 and CM5, despite the significantly larger volume of CM3 and particularly CM5. As mentioned already this might have been caused by the electrostatic repulsion between the negatively charged carboxydextran matrix and the negatively charged pepsin. In contrast, the lack of a significant electrostatic charge on the polymer layer allows the negatively charged protein to approach the surface. The polymer-coated surface performed better than CM3 for most of the tested proteins with the exception of carbonic anhydrase. This might be

due to the fact that this latter enzyme contains a zinc ion, which might promote electrostatic attraction to the negatively charged carboxydextran matrix. CM5 showed in all cases, with exception of pepsin, higher binding capacity than the polymer-coated surfaces.

In conclusion polymer-coated surfaces are particularly advantageous when protein with low p*I* needs to be immobilised onto sensor surface. Another important advantage of the 3-D polymer-coated chips would be the cost. All the reagents involved on the synthesis of the polymer are affordable, in contrast to CM3 and CM5, which are relatively expensive.

4.4. Conclusions

The work presented in this chapter describes the development of a new low-cost, fast and simple method for covalent immobilisation of proteins and other amino-containing biomolecules by employing an inexpensive and simple thioacetal-based polymers. These polymers offer a number of advantages. They contain groups that promote self-assembling on a metal (preferable noble) transducer surface. They also contain groups that are able to bind amino-containing substances without any additional activation. Three different categories of polymers were synthesised and after extensive optimisation of the composition, a 3-D polymer (based on pol. 20) was obtained. Other desirable properties of the 3-D polymer are both the opportunity to immobilise appropriate amount of ligands and the easy accessibility of binding sites given by the tridimensional structure. Immunosensors developed on 3-D polymer-coated surface showed high sensitivity for the target analyte. In fact detection limits of 10 ng ml^{-1} and 0.05 ng ml^{-1} were obtained for microcystin-LR and PSA, respectively. Polymer-coated surfaces possess also higher loading capacity for proteins with low p*I*,

when compared with commercial Biacore chips CM3 and CM5. All these advantages in combination with, the simple and inexpensive synthesis make this new polymer very promising for the development of low-cost, easy to prepare and sensitive biosensors.

CHAPTER 5

POLYMER APPLICATION FOR PSA DETECTION IN SERUM

5.1 Introduction

Specific detection of proteins in serum is very challenging due to the complex nature of the matrix, which contains a high content of many different compounds. Thus in the current work the developed 3-D polymer described in Chapter 4 was applied and the possibility to use it as a material to develop sensors for PSA detection in serum was studied.

As it has been described in previous chapters, biomolecules immobilisation on the polymer is based on the well known reaction between primary amines and thioacetal containing matrix (Simons and Johnson, 1978; Piletska *et al.*, 2001). A prerequisite necessary to detect analytes in complex samples is that the matrix used for the immobilisation of the receptor should produce a low level of non-specific interactions. This is because real samples such as serum are very complex and contain thousands of different molecules (Kusnezow and Hoheisel, 2003). A low level of nonspecific binding is usually achieved by inclusion of polar molecules such as polysaccharides or polyethylene glycol derivatives into the immobilisation matrix (Masson *et al.*, 2005).

In this study different strategies were investigated in order to reduce the non specific adsorption of serum proteins on polymer surfaces with immobilised antibodies. These techniques include addition of polymerisable Polyethylene Glycol (PEG) in the polymer composition, blocking with a charged hydrophilic amino acid (aspartic acid) or amino-PEG and inclusion of a surfactant like P20 into the analytical system. For comparison the same techniques were also tried on Biacore commercially available CM5 carboxydextran surfaces. In the last part of this work, a SPR sensor based on a

sandwich assay was developed on the 3-D polymer and optimised to detect PSA in serum.

In fact, a sandwich immunosensor, which involves two primary antibodies binding two different epitopes of the antigen PSA, can be used without any prior activation on sensor surfaces modified with the polymer (Fig. 5.1). In this sensor format, after the immobilisation of the first antibody, which is a capture antibody (Ca-Ab) on the 3-D polymer modified chip, PSA contained in a real sample (blood or serum) can be passed and bound to the sensor. This will be followed by the specific binding of a second antibody, a detection antibody (D-Ab), which is capable to bind PSA in a different epitope than C- Ab. This detection reaction can be performed with D-Ab dissolved in a buffer solution. The recording of the specific sensor signal takes place in this last step avoiding problems of non specific binding. In addition, with this sensor format, there is an enhancement of the response due to the big size of D-Ab (165 kDa) comparing with PSA (33 kDa). When a sandwich format was applied in this work and detection of PSA was carried out in real samples, very encouraging results were obtained, demonstrating that the novel matrix developed in this work could be successfully applied for clinical applications.

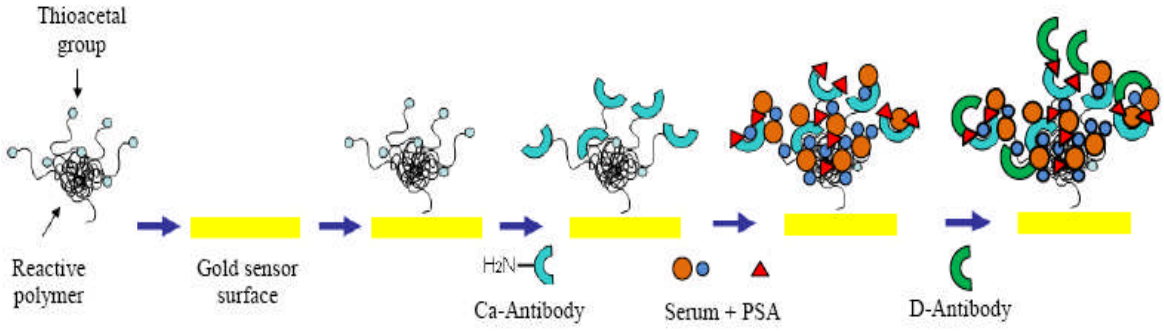


Fig. 5.1. Schematic representation of anti-PSA Ab immobilisation on the 3-D polymer-coated gold surface and all the steps of the detection of PSA in serum by applying a sandwich assay.

5.2. Materials and Methods

5.2.1. Reagents- Materials

Most compounds were obtained from commercial distributors and were of analytical or HPLC grade. Triethylamine (TEA), bovine serum albumin (lyophilized powder) human serum (male) and IgG from bovine serum were purchased from Sigma (UK). Monoclonal mouse anti-PSA capture antibody (anti-PSA Ca-Ab), monoclonal mouse anti-PSA detection antibody (anti-PSA D-Ab) and PSA were purchased from Ab-Serotec (UK). Allyl thiol (AT), N,N -bis(acryloyl)cystamine (BAC), o-phthaldialdehyde (OPA) were purchased from Fluka (UK). 2-Hydroxyethyl methacrylate (2-HEM), ethylene glycol dimethacrylate (EGDMA), 1,1,1-tris(hydroxymethyl)propan trimethacrylate (TRIM) and 2-benzyl-2(dimethylamino)-4'- morpholinobutyrophenone (BDMB) were purchased from Aldrich (UK). Ethanolamine, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), N-hydroxysuccinimide (NHS), surfactant P20 (10% v/v), NaOH solution (0.2 M), 10

mM glycine- HCl pH 2.0, SIA Kit Au and CM5 chips were purchased from Biacore (Sweden). Solvents were of analytical or HPLC grade and supplied by Acros Organics (UK). The water was purified by a Milli-Q water system (Millipore, Bedford, MA, USA) and all the reagents used for Biacore experiments were filtered using a 0.22 μm filter from Phenomenex® (UK).

5.2.2. Polymer synthesis

The synthesis of the 3-D polymer used for the development of the sandwich sensor was the same as described in Chapter 4. Very briefly the polymer was synthesised by mixing 2.0 mmol (260 mg) of 2-HEM, 0.3 mmol (60 mg) of EGDMA, 1.5 mmol (507 mg) of TRIM, 1.0 mmol (134 mg) of OPA, 2.0 mmol (150 mg) of AT, 0.1 mmol (26 mg) of BAC, 0.5 mmol (180 mg), BDMB (initiator) and DMF (5 mL) as solvent. A small amount of TEA (40 μl) was added to the monomer mixture and it was thoroughly purged with argon for 5 minutes. Polymerisation was initiated by placing the mixture under a high intensity Hönle 100 UV lamp (intensity 0.157 W cm^{-2}) for 20 minutes. The synthesised polymer was then precipitated from DMF by adding 20 ml of water and washed several times with methanol. Another 3-D polymer was also synthesised with the same protocol, but with the addition of PEG into the polymer composition. In this case 0.2 mg and 0.5 mg of PEG, which represents the 20% and 50% respectively of the total mass of the polymerisable material, was used.

5.2.3. Treatment of gold chips – gold surface modification

Sensor chips, SIA Kit Au (Biacore, Sweden) were used to assess the ability of polymer-coated surfaces to bind proteins. SIA Kit Au chips were cleaned for 3

minutes using oxygen plasma at 40 W in a plasma chamber (Emitech, UK). Polymer was self-assembled onto SIA Kit Au by immersing chips in 5 ml acetone/ethanol 50/50 (v/v) containing 10 mg ml^{-1} of polymer for 24 h. The polymer-coated gold chips were rinsed thoroughly with acetone/ethanol, dried with nitrogen and assembled onto the holder. In order to be able to reuse Biacore chips, the sensors were left in a methanol solution for one day to dissolve the glue and remove the holder. The chips were then cleaned with oxygen plasma at 40 W and considered suitable to be reused, only after keeping them into a THF solution to remove any further contaminants deriving from the holder.

5.2.4. SPR experiments

All the SPR experiments were performed with Biacore 3000 (Sweden) at 25^{o}C.

5.2.4.1. *Evaluation of serum adsorption on sensor surfaces modified with antibodies and blocking agents.*

Biomolecules and some blocking agents were immobilised on 3-D polymer sensor surfaces and the immobilisation monitored by Biacore 3000 in continuous flow system. The change observed in Biacore response units (RU) is directly proportional to the change of surface mass; 1 RU in general is approximately equivalent to 1 pg mm^{-2}. The antibody applied to study the performance of the polymer-modified surface for analyte detection in complex matrix, like serum, was mouse monoclonal anti-PSA antibody (anti-PSA Ab and the blocking agents applied to reduce the non specific adsorption from serum were BSA, Ethanolamine (ETA), amino- PEG and aspartic acid. For comparison, the non specific adsorption of serum on CM5, which is a Biacore carboxymethylated dextran chip, was also assessed by applying the same

biomolecules and blocking agents. For experiment on CM5 an activation step performed with EDC/NHS (Fagerstam et al., 1992) was applied, while polymer-coated surface did not require any activation. Activation of CM5 was performed by injecting 30 µl of 0.2 M EDC/0.05 M NHS with a flow rate of 5 µl min^{-1}. Typically proteins (Ab, BSA) immobilisation was carried out on polymer-modified surfaces by injecting 150 µl of 25 µg ml^{-1} of Ab and 100 µg ml^{-1} of BSA in 0.01M phosphate buffer saline (PBS), pH 7.4 with a flow rate of 10 µl min^{-1}. Immobilisation of Ab and BSA on CM5 was performed with the same procedure, but using 0.05 M Na-acetate buffer, pH 5.0 instead of PBS. After protein coupling the remaining active groups on both surfaces (polymer modified-chips and CM5) were deactivated by injecting 30 µl of 1 M ETA pH 8.5, with flow rate 10 µl min^{-1}. In another set of experiments the sensor surfaces were directly modified with different blocking agents by injecting them until saturation. The degree of adsorption of serum onto all the resulting surfaces was assessed by injecting 50 µl of 10% human serum diluted in PBS pH 7.4 with a flow rate of 10 µl min^{-1}. Furthermore serum adsorption on polymer-modified and CM5 chips was also studied after antibody immobilisation (and blocking with ETA) by including the surfactant P20 (0.005% v/v) in both serum solution and running buffer.

5.2.4.2. PSA detection in PBS with direct and sandwich detection.

The immobilisation of anti- PSA C-Ab on polymer-modified chips was performed by injecting 150 µl of 25 µg ml^{-1} of Ab solution in PBS buffer with pH 7.4 with a flow rate of 10 µl min^{-1}. After Ab coupling the remaining active thioacetal groups were deactivated by injecting 30 µL of 1 M Ethanolamine pH 8.5 and several

injections (4-6) of 30 µl of BSA (200 µg ml^{-1}) in PBS pH 7.4 with a flow rate of 10 µl min^{-1} until saturation. After immobilisation of anti- PSA Ab on polymer chips and blocking, detection of PSA was performed in PBS buffer pH 7.4 with 20 µg ml^{-1} BSA and 0.005% (v/v) surfactant P20. This buffer was also used as running buffer for both direct and sandwich detections. For direct detection, PSA was diluted in the running buffer in concentrations ranging from 0.1 to 1000 ng ml^{-1} and injected for 5 minutes, with a flow rate of 10 µl min^{-1}. For detection of PSA by sandwich format, following each injection of PSA, performed as described for direct detection, and after waiting 5 minutes for stabilisation of the baseline, the detection signal was recorded by injecting, 50 µl of anti-PSA (D-Ab) 10 µg ml^{-1} prepared in running buffer with a flow rate of 10 µl min^{-1}. A reference channel containing polymer with IgG (from Bovine Serum) immobilised was used for assessment of binding specificity. Regeneration was carried out by injecting 10 mM NaOH for 30- 60 s both for direct and sandwich format with a flow rate of 30 µl min^{-1}. In this work the LOD was determined as the first injected concentration showed a response equal to that obtained on the reference channel plus three times the STD. The response on reference channel is considered as noise or non specific binding. LOD should not be confused with the limit of quantification, LOQ, which is calculated as the first concentration, which shows a response equal to that of the reference channel plus 10 times the STD (Long and Winefordner, 1983; IUPAC).

5.2.4.3. PSA detection in serum with sandwich format

The running buffer for PSA detection in serum was PBS pH 7.4 containing 0.005% (v/v) P20. For the experiments human serum was diluted five times in running buffer (20% serum). This solution was then used to prepare samples spiked with PSA in

concentrations ranging from 0.1 to 500 ng ml^{-1}. Firstly anti-PSA C-Ab were immobilised on polymer-modified chips as described above. Then diluted human serum samples spiked with PSA were injected over the immobilised C-Ab for 5 minutes with a flow rate of 10 µl min^{-1}. Afterwards the detection of analyte was performed by injecting 50 µl of 10 µg ml^{-1} anti-PSA (D-Ab) with a flow rate of 10 µl min^{-1}. The signal was corrected by subtracting the values obtained after flowing the same concentration of D-Ab over the reference channel. This reference channel consisted on a sensor surface with immobilised C-Ab, on which only diluted serum without added PSA was injected. In other experiments, the specificity of the reaction was assessed by immobilising on the reference channel a different antibody (non specific for PSA) which was then exposed to the same procedure of blocking and detection as for the working channel.

5.3. Results and discussion

5.3.1. Immobilisation of antibodies and blocking agents on sensor surface

The compounds applied on sensor surface in order to study the non specific adsorption of serum were anti-PSA C-Ab, BSA, aspartic acid, ethanolamine and amino-PEG. The sensor surfaces were the 3D-polymer and Biacore carboxydextran CM5 chips. The amount of compounds immobilised on the two surfaces is illustrated in Table 5.1.

Table 5.1. *The amount of compounds immobilised on 3-D polymer and CM5 biacore carboxydextran surfaces. Immobilisation of BSA (100 μg ml^{-1}) and anti-PSA Ca (25 μg ml^{-1}) occurred with 2 injections of 50 μl each and flow rate 10 μl min^{-1}. For the other compounds sequential injections of 50 μl with the same flow rate were applied until surface saturation was observed. The immobilisation was performed in PBS buffer pH 7.4 and acetate buffer 50 mM pH 5.0 for polymer surface and CM5 correspondingly.*

Compound	***Immobilised ligand on Polymer surface Biacore (RU) (%)[a]***	***Immobilised ligand on CM5 Biacore (RU) (%)[a]***
BSA	1323.7 ± 166.8	10126.3 ± 951.9
Anti-PSA Ca	2563.1 ± 417.8[b]	13349.0 ± 2.2%
Aspartic Acid	1260.9 ± 148.8	---
Amino-PEG	1397.9 ± 381.6	156.1 ± 51.4
ETA	774.2 ± 115.4	147.9 ± 43.8

[a] *Standard deviation was calculated from a set of four experiments.*
[b] *Standard deviation was calculated from a set of twenty six experiments.*

It is well known that Biacore CM5 possesses a carboxydextran surface with thickness around 5 nm, which is then swollen to around 100 nm in presence of aqueous solutions. Therefore once the buffer solution runs over surfaces of CM5 the carboxydextran thickness becomes around 100 nm. Another important property of CM5 is the hydrophilicity and the presence of negative charges, which makes the surface repellent to many proteins and biomolecules. Table 1 shows that higher immobilisation of aspartic acid, amino PEG and ETA were obtained on polymer surfaces than on CM5. This is due to the electrostatic repulsion between the negatively charged matrix on CM5 and the negative charged groups of the compounds when immobilisation is performed at pH 4.5-5.5. On the contrary, as it was expected higher binding capacity of CM5 was observed for BSA and anti-PSA C-Ab, because of the larger surface volume.

To improve the performance on polymer-surfaces, polymerisable PEG was included in the polymer composition at 20 and 50% of the total mass of polymerisable material and the study repeated. The difference on the amount of immobilised protein and blocking compounds on these new polymers was negligible (data not shown).

5.3.2. Effect of proteins, blocking agents and PEG polymer on non specific interaction of serum with the sensor surfaces

The non specific adsorption on sensor surfaces of complex matrix such as serum is a limiting factor for direct analyte detection in real samples. Thus, various methods were tested in order to reduce the non specific interaction of serum with the sensor surfaces. These included inclusion of polymerisable PEG in the polymer and the application of blocking agents. The reduction of non specific serum adsorption on PEG-polymer was ca. 37 % when compared with the original polymer composition. The total response for serum on PEG polymer was, however, still over 1000 RU and therefore too high for direct measurement of PSA in real samples.

As a result, several blocking methods were tested. 3D-Polymer and CM5 chips were used for comparison, and the results are summarised in Table 5.2. As it can be seen in the Table, serum shows high adsorption on the 3-D polymer matrix, whereas the adsorption on Biacore CM5 was low. This could be due to the hydrophilic surface of this latter, which repels most of the serum components. It is however clear that a large percentage of non specific adsorption of the serum, both on CM5 (980.7 RU) and polymer surfaces (376.9 RU) occurs on the antibodies themselves. This is demonstrated by the increased serum adsorption on CM5 after immobilisation of anti-PSA Ca. In addition, the inclusion of P20 both in running and immobilisation buffers

reduced significantly the serum adsorption on Ab immobilised 3-D polymer surfaces, whereas it did not significantly improve non specific adsorption on Ab immobilised on CM5 chips.

These findings lead us also to the conclusion that special care should be taken to choose the material immobilised on a reference surface to assess the non specific binding, while measuring real samples. For example if the reference surface is blocked with ethanolamine or BSA the non specific serum adsorption would be low, whereas it could be significantly higher if a different biomolecule such as an antibody is used as control. Also the adsorption of matrix components can differ from antibody to antibody and these can lead to positive or negative error in analyte determination. Thus, it is reported in Biacore Sensor Surface Handbook (2003) that the choice of the biomolecules, which are applied for working and reference channel, can have a significant effect on the assessment of the non-specific binding. When detection of analytes is performed in complex matrices like serum, immobilisation of antibody fragments is preferable to intact antibody. In fact, the application of fragments as receptors can reduce the binding of matrix components on to the sensor surface (Biacore Sensor Surface Handbook, 2003).

Table 5.2. *Biacore responses to serum of 3-D polymer and CM5 chips with different surface blocking methods. The immobilisation of serum was performed at a flow rate of 10 µl min^{-1} for 5 minutes. The immobilisation of BSA (100 µg ml^{-1}) was performed with 2 injections of 7.5 minutes with flow rate of 10 µl min^{-1}. Blocking with ethanolamine (ETA) was performed with 1 injection of ETA 1 M pH 8.5 at 10 µl min^{-1} for 3 minutes. Immobilisation of the small size compounds was performed until saturation of surface. The immobilisation on polymer chips was performed with PBS, pH 7.4 and on CM5 with Na-acetate buffer 50 mM pH 5.0. The experiments were repeated 3 times.*

Immobilised blocking ligand	**Response (RU)**			
	Serum (1/10) in PBS on CM5 chips	Serum 1/10 in PBS on 3-D polymer chips	Serum 1/10 in PBS with 0.005% P20 on CM5 chips	Serum 1/10 in PBS with 0.005% P20 on polymer surface
ETA	96.4 ± 9.5	2160.4 ± 147.3	33.4 ± 8.2	1106.9 ± 27.8
Amino-PEG	125.9 ± 10.0	1685.5 ± 77.1	26.1 ± 13.6	847.6 ± 87.1
Aspartic Acid	Low pI	1366.2 ± 102.6	Low pI	712.0 ± 62.2
BSA	102.1 ± 11.9	1095.7 ± 73.6	15.7 ± 4.1	431.0 ± 25.7
Anti PSA-Ab	1110.5 ± 61.4	1570.3 ± 113.5	980.7 ± 38.4	376.9 ± 91.4

5.3.3. Detection of PSA in PBS

Detection of PSA both by direct and sandwich formats was carried out. For the sandwich format a C- Ab and a D-Ab, which bind different parts of the antigen, were used as receptor and detection element correspondingly. The running buffer during detection was PBS containing 10 µg ml^{-1} of BSA. The inclusion of BSA in running buffer decreased dramatically the non specific binding on the reference channel and also helped with detection of the lowest concentrations (data not shown) by preventing protein adsorption to vials and injection system. Moreover BSA is also known as stabiliser of proteins helping in maintaining the protein bioreactivity (Kyo *et al.*, 2005). Fig. 5.2 shows the calibration curves obtained both by direct and

sandwich formats injecting increasing PSA concentrations prepared in PBS. The limit of detection, LOD, for PSA in PBS was found to be 0.1 ng ml^{-1} in both detection formats. This LOD is by far lower than the 10.2 ng ml^{-1} reported in literature by Cao and co-workers (Cao *et al*., 2006) and still better than the method developed by Besselink and co-workers (Besselink *et al.*, 2004), who achieved LOD of 0.15 ng ml^{-1} of f-PSA in PBS buffer containing 3% BSA, by amplifying the signal with gold nanoparticles. Possible reason for achieving such a low LOD could stand to the application of non-modified antibodies. In fact chemical modifications of antibodies can cause complete or partial loss of their bioreactivity due to conformational changes during conjugation (Hermanson, 1996). In addition for every immunosensor the inherent affinity of the antibody for the analyte is a key factor for the sensitivity of the detection system. Another reason for achieving such a low LOD could also be the application of the 3-D polymer for antibody immobilisation, since it has been demonstrated in the past that flexible porous and non-porous polymer films perform better than homogeneous flat surfaces (Masson et al., 2005). This can be attributed to an improvement of protein diffusion in the polymer matrix, together with partial protection of protein structures from unfolding processes. Rigid or solid surfaces often cause irreversible denaturation of the bound proteins (Su *et al*., 1998).

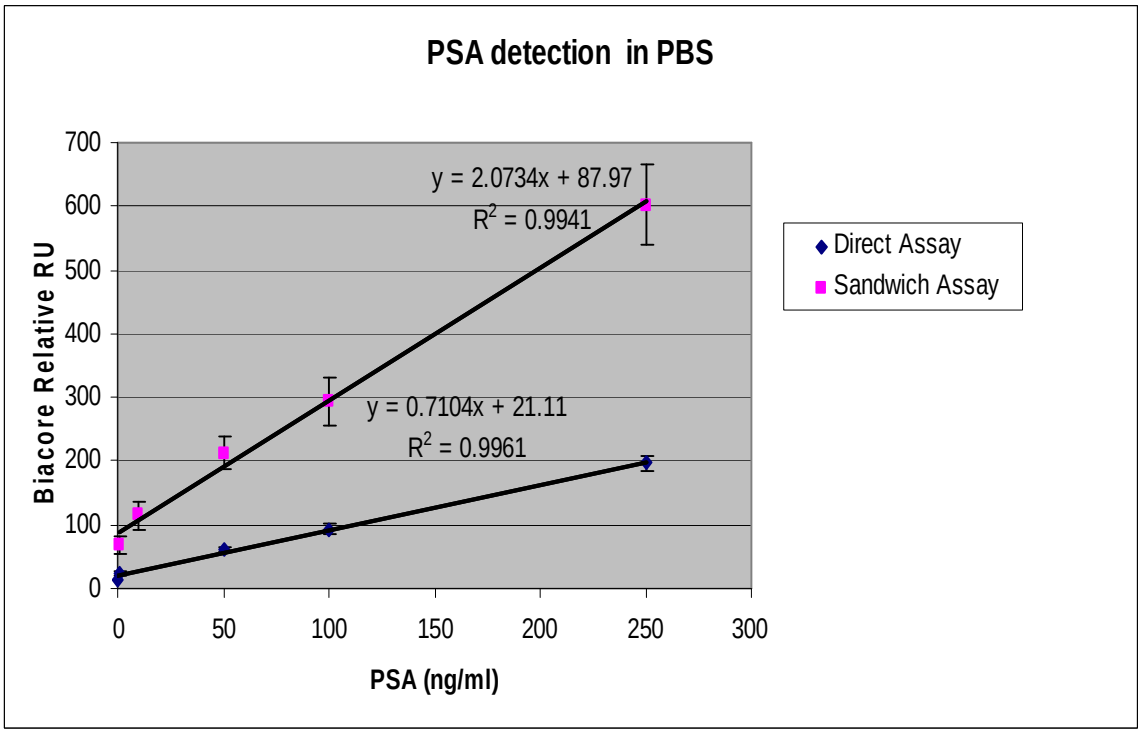


Fig. 5.2. *Calibration curves of PSA detection in PBS in concentration ranging from 1 to 250 ng ml^{-1}obtained by direct and sandwich formats. The responses and standard deviations were calculated from a set of 4 measurements (n=4). Error bars represent the STD of Biacore response (RU) for each concentration.*

The application of a big molecule like antibody (D-Ab) as detection element can produce higher non specific binding (NSB) on reference surface, but at the same time higher signal on working channel. In this work the values of NSB were 3.7 ± 3.1 RU (n = 6) and 9.5 ± 4.2 RU (n=6) for PSA and D- Ab respectively. As it has been demonstrated in the literature, the main advantage of using a sandwich detection format is the significant signal enhancement of the analyte response after the injection of detection antibody. Here the signal obtained with the sandwich format was 2.5 - 3 times higher as compared with the direct detection and consequently a higher sensitivity was achieved, see Fig. 5.2. As it is depicted in this figure the direct detection showed a linear regression equation of y = 0.7104 x + 21.11, whereas the same equation for sandwich format was y = 2.0734 x + 87.97. The equations

confirmed the higher sensitivity of the sandwich detection, since according to the slopes of the two curves the sensitivity was improved by a factor of 2.92. The linearity of both curves can be considered satisfactory for sensor applications, as R^2 values of 0.9961 and 0.9941 were obtained for direct and sandwich immunoassay correspondingly. Regarding the calibration curve of both direct and sandwich detection, at concentrations higher than 250 ng ml^{-1} the curve approached saturation and there was significant deviation from linearity (data not shown) .

The sensograms obtained for PSA detection in concentrations ranging from 1.0 to 250.0 ng ml^{-1} by using the sandwich format are reported in Fig. 5.3. An important step of sandwich detection format is the choice of the right time for the injection of D-Ab. In fact this injection should be performed at a precise time after the antigen detection. The reason is that early injection of D-Ab during analyte dissociation will lead to errors and high standard deviations especially for low concentrations, as during the antibody injection, association of D-Ab and dissociation of antigen will take place at the same time.

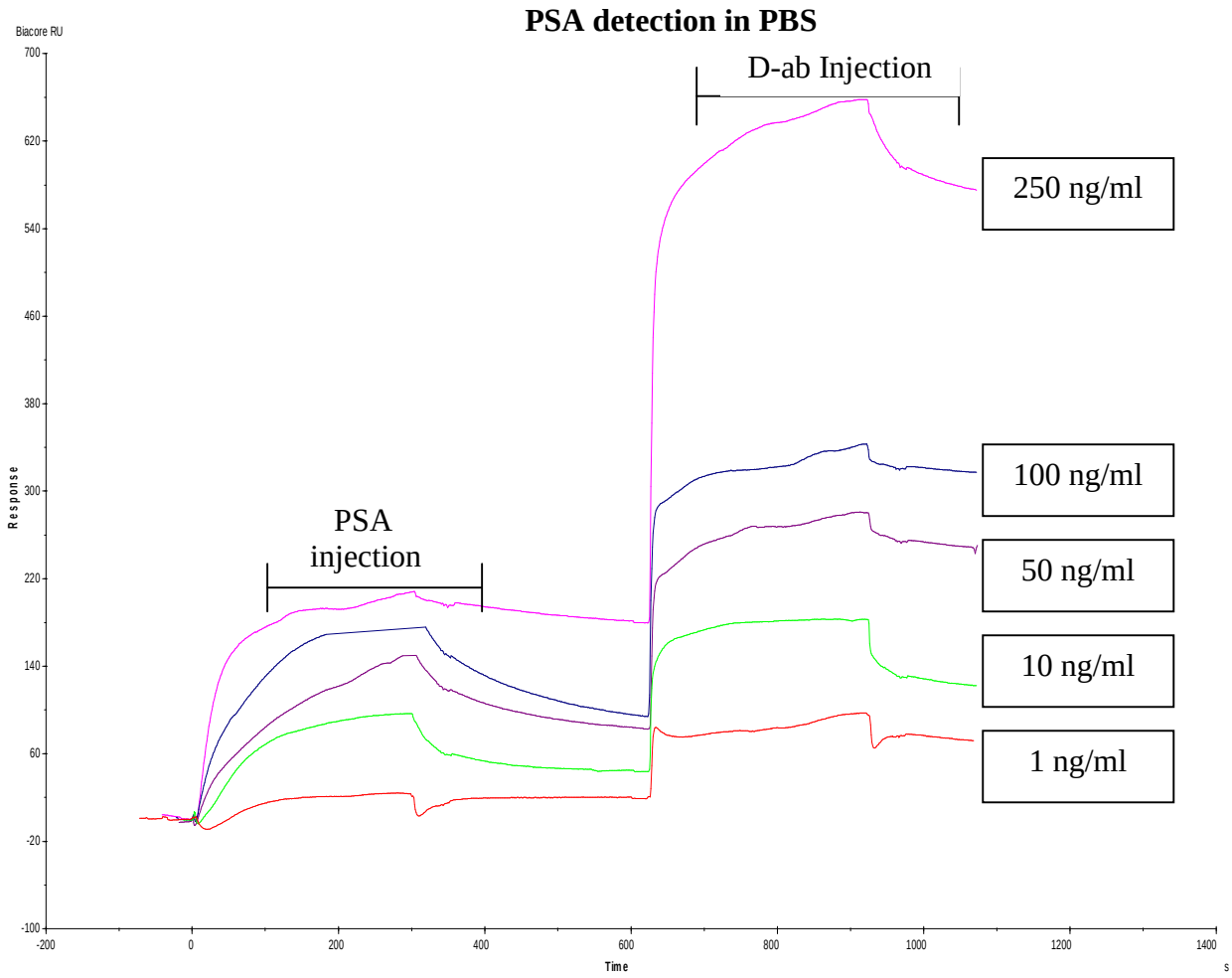


Figure 5.3. *Detection of PSA by sandwich format in concentrations ranging from 1 to 250 ng ml^{-1}. Firstly the antigen is injected on a 3-D polymer surface where anti-PSA (c-Ab) was immobilised. After a reasonable time allowing for antigen dissociation, anti-PSA (D-Ab), which recognises a different epitope of the analyte, is injected and the detection signal is enhanced.*

The regeneration of sensor surfaces after the immunoreaction is of high importance since it gives indication of the specificity of binding. In addition, if the regeneration is successful the sensor could be reutilised for a further measurement, which makes the immunosensor applications more cost effective. Thus on Fig 5.4 the regeneration step perfomed at the end of the sandwich assay by injecting 10 mM NaOH for 30-60 s is illustrated.

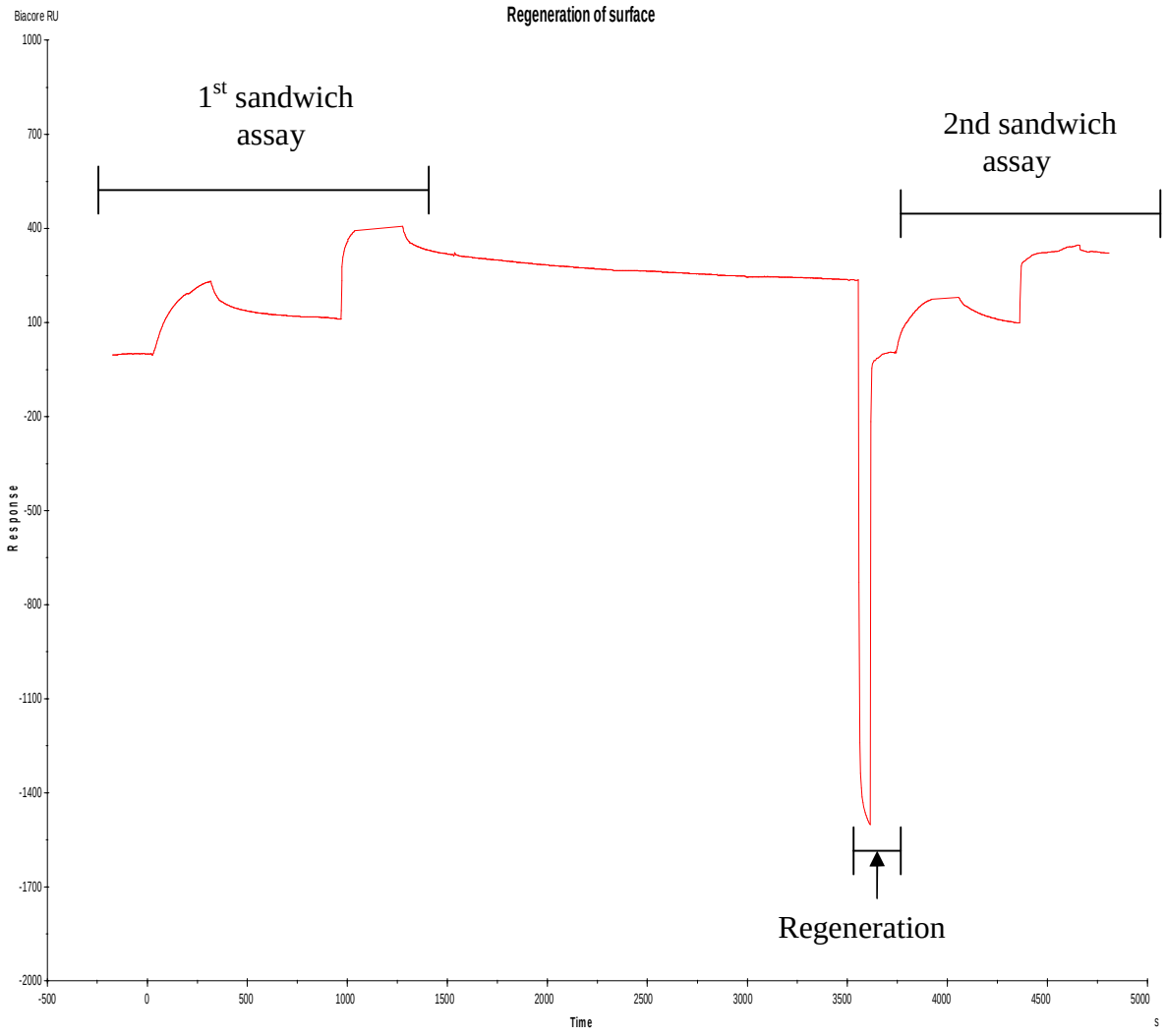


Figure 5.4. *Regeneration of sensor surface after detecting 100 ng ml^{-1} of PSA by sandwich format. The regeneration was performed by injecting 10 mM NaOH for 30 s, with a flow rate of* 30 µl min^{-1}. *After regeneration the baseline returns to values similar to the beginning of the immunoassay. The assay performance in the second binding cycle is also similar to that of first cycle.*

The immunoassay showed similar response for 3 regeneration cycles performed as explained above.

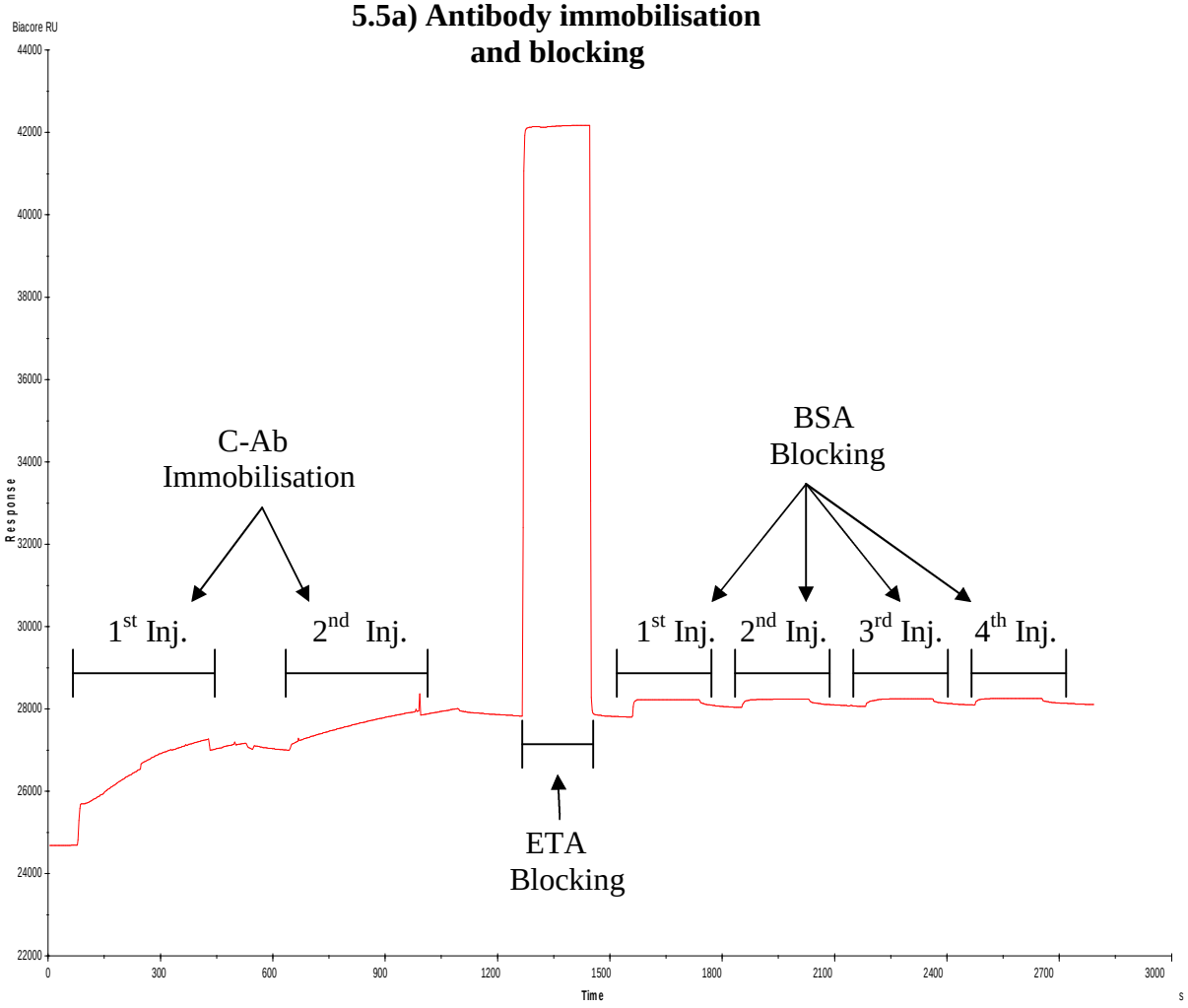
5.3.4. Detection of PSA in serum

The detection of PSA in serum is difficult and challenging. As it has been previously mentioned the presence of high amount of proteins in serum makes the detection of any analyte very difficult. In order to achieve this, as it was described in section 5.3.2, different strategies were employed including the addition of PEG in polymer synthesis, blocking with amino-PEG and amino acids as well as the inclusion of surfactant in running buffer. Unfortunately none of these methods were able to reduce the non specific adsorption of serum components on the polymer surface at a level suitable for direct detection and determination of PSA in clinical samples. Thus it was decided to perform PSA detection with a sandwich format using a detection antibody, D-Ab, which recognises a different epitope on the antigen than the capture antibody C-Ab, the sensing element immobilised on 3-D polymer surfaces. The application of a second antibody as a detection element in this case is advantageous, since the 'noise', caused by adsorption of serum components on sensor surface during injection of the analyte, does not influence the detection step. PSA detection was performed in 20% serum (diluted in PBS with 0.005% P20) as it was described in Material and Methods. The use of a higher percentage of serum is not recommended for Biacore applications, since it can cause blockage of microfluidic system and problems for further applications. The use of 20% serum for PSA detection is a step closer to real samples analysis, as in most of reported applications in literature the PSA was added in 10% serum solutions (Cao *et al.*, 2006). There is, however, one application, reported by Yu and his colleagues (Yu *et al*., 2004), where the PSA was detected directly in human

plasma, but in this example the sample was delivered over the sensor surface using a homemade glass flow cell.

The measurement procedure for PSA detection in serum using the sandwich format is depicted in Fig. 5.5a and 5.5b. As it is shown in Fig. 5.5a Ab immobilisation was carried out with two injections, in order to obtain a higher amount of antibody immobilised on sensor surface. Afterwards ETA and BSA were applied to block the unreacted binding sites and provide a better coverage of the relatively hydrophobic surface (modified with 3-D polymer). This showed to minimise the non specific adsorption of analyte and detection antibody D-Ab. The injection of PSA and D-Ab is shown in Figure 5.5b. As it has been previously mentioned in 5.3.3. the injection of D-Ab should be performed after stabilisation of the baseline to avoid errors in detection by having at the same time association of antibody and dissociation of PSA or serum proteins from the surface.

5.5a) Antibody immobilisation and blocking



5.5b) PSA detection in serum

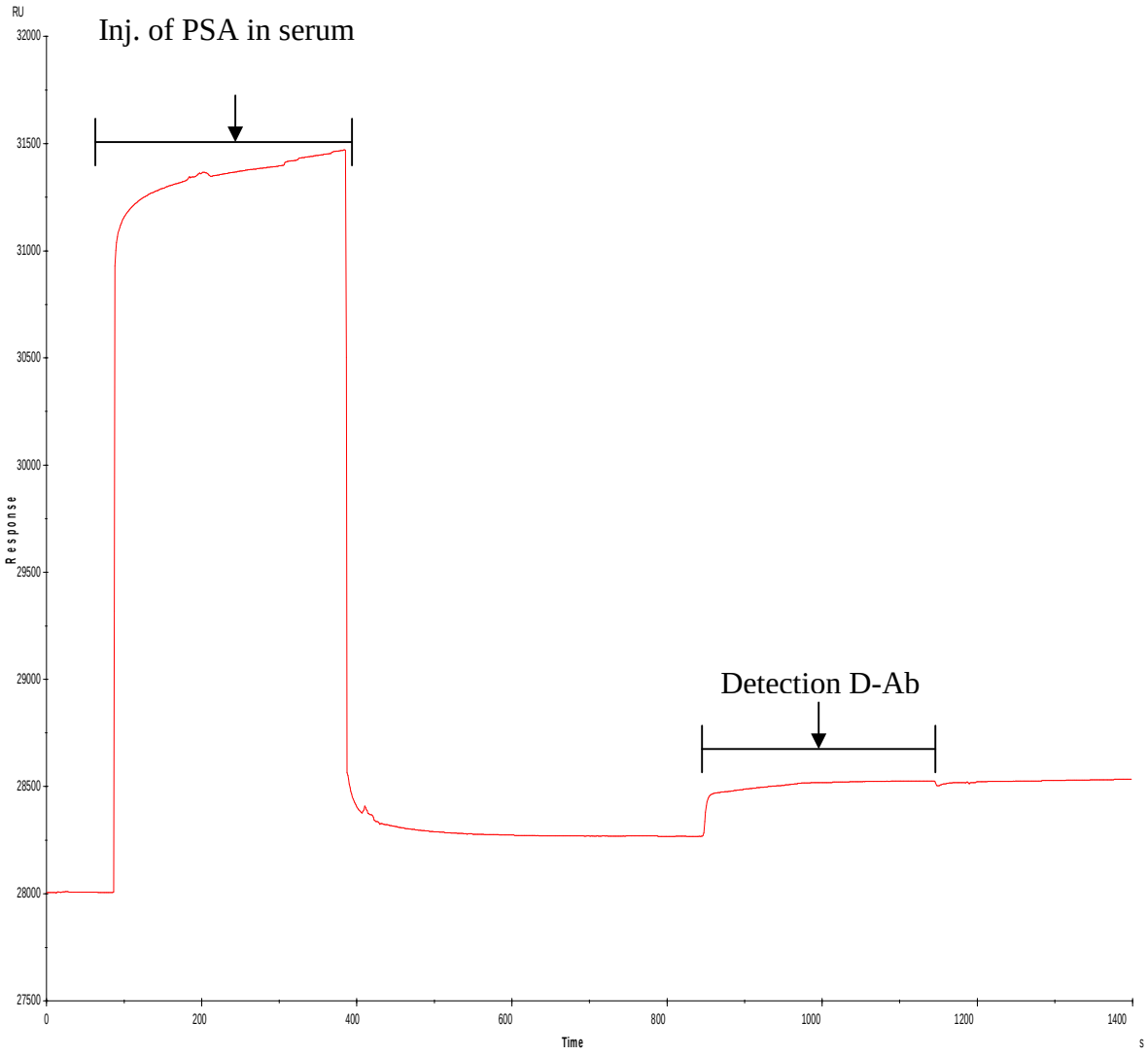


Fig 5.5. *Biacore sensograms illustrating detection of 100 ng ml^{-1} of PSA by sandwich format. (a) Surface preparation for PSA detection. The first two injections correspond to the C-Ab immobilisation following by blocking of unreacted binding sites by one injection of ETA and four injections of BSA for 180 s; (b) PSA detection in serum. Firstly the 20% serum solution spiked with PSA 100 ng ml^{-1} is injected. Afterwards detection is performed by injecting for 5 minutes 10 μg ml^{-1} of D-Ab prepared in running buffer (PBS with 0.005% P20).*

Several PSA concentrations ranging from 0.1 to 500.0 ng ml^{-1} were studied in order to determine the detection limit (LOD) and the correlation between PSA concentration

in serum and the resulting Biacore response after injection of D-Ab. The biacore responses (RU), obtained after injecting the detection element (D-Ab), *vs* PSA concentrations ranging from 1.0-250 ng ml^{-1} prepared in 20% are shown on Fig, 5.6a. As it is illustrated in this curve, the linear range for PSA determination in serum did not include all the concentrations tested, but was between 5.0 and 100 ng ml^{-1} (Fig. 5.6b). The slope of the curve was reduced from 2.07, for detection of PSA in PBS (Fig. 5.2) to 1.64, which indicates a reduction in sensitivity. This result is in agreement with the work of Cao and colleagues, who also observed reduction in sensitivity and slope when the determination of PSA-ACT was performed in serum (Cao *et al.*, 2006). In this work, the lowest concentrations of PSA could not be measured with high confidence due to the non-specific adsorption of D-Ab recorded on the control surface, which gave a signal of 12.6 ± 6.1 (n=6) RU. This control (reference) channel was prepared by immobilising C-Ab on 3-D polymer followed by blocking as in working channel. The non specific binding to this channel was then assessed by injecting D-Ab, after injection of 20% serum without PSA. The LOD of the sensor which was 5.0 ng ml^{-1} was calculated as the first concentration showing a signal higher than the average response on this control channel plus three times the STD. In reality a small proportion of the signal recorded on this control channel might have been specific, as male human serum was used for the experiments and some PSA might have been present. The concentration of the analyte in non spiked serum, however, is expected to be lower than 0.6 ng ml^{-1} as PSA level for healthy men varies between 0.1- 3.0 ng ml^{-1} and the serum samples were diluted 5 times. Nevertheless the sensor values obtained for PSA spiked samples were corrected by subtracting the response of the reference channel. As it is shown on Fig. 5.6a, at PSA concentrations higher than 100 ng ml^{-1} the calibration curve showed deviation from linearity, most

likely because of saturation of Ab binding sites. In addition, by comparing the calibration curves obtained by sandwich format performed in serum (Fig.5.6a) and in buffer (Fig. 5.2), it can be noticed that saturation in serum takes place earlier than in buffer and consequently the sensitivity of the sensor in serum is lower comparing with the sensor in PBS. This could be due to the lower accessibility of PSA epitope for binding to D-Ab during detection, caused by adsorption of other serum components.

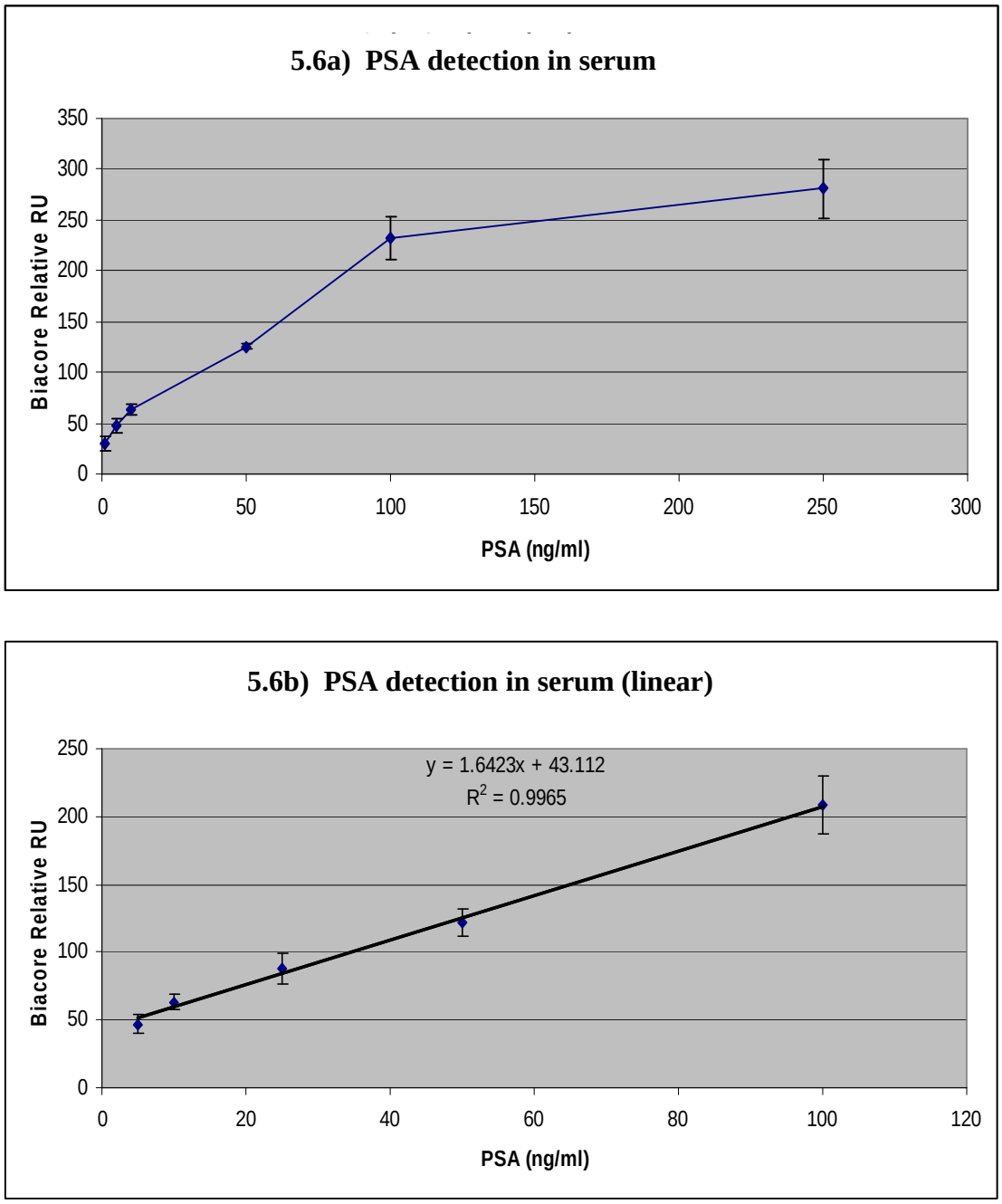


Fig. 5.6. *(a) Calibration curve obtained by plotting Biacore response (RU) after D-Ab injection vs PSA concentrations added in serum solution;. (b) Linearity range of calibration curve. Error bars represent the STD of Biacore responses (RU) for each PSA concentration (spiked in 20% serum) and it was calculated from a set of 3 experiments.*

The results obtained for PSA detection in serum are promising and a step forward on the development of a low cost, simple and fast immunosensor suitable for testing clinical samples. The LOD for PSA detection in serum was 5.0 ng ml^{-1} can be

considered satisfactory compared with similar applications reported in literature. For example Cao and co-workers, (Cao *et al.*, 2006), by applying sandwich format, have achieved a detection limit of 18.1 ng ml^{-1}. The lower detection limit achieved in this work could be a result of the application of the 3-D polymer and the relatively high affinity of the antibody applied for PSA detection, as it has been already explained in section 3.3.1. A very low LOD, at femtomolar level, was achieved by Yu (Yu *et al.*, 2004), but in this case detection was performed by surface plasmon fluorescence spectroscopy (SPFS) after labelling the D-Ab with a fluorescent dye. The LOD of 5.0 ng ml^{-1}, obtained here using 20% serum solutions, would correspond to 25.0 ng ml^{-1} of PSA in pure serum. The concentration range of clinical interest is 1.0-10.0 ng ml^{-1}. Therefore it would be very important to be able to develop a sensor where serum could be injected without dilution, since dilution creates the demand for a lower LOD and a higher sensitivity. One way to achieve this would be either improving the affinity of the antibodies applied for the reaction or developing haptamers or other synthetic receptors with higher affinity and specificity. In addition also the matrix used for receptors immobilisation in this work, the 3-D polymer, could be further improved to achieve higher surface capacity and at the same time reduce the non specific adsorption, which would result in sensors with higher sensitivity. Other methods that researchers are currently using for improving immunosensors sensitivity (signal enhancement) involve gold colloidal nanoparticles (Besselink *at al.*, 2004), the application of SPFS (Yu et al., 2004) or enzyme precipitation, which is a double enhancement assay. By applying double enhacement assay the detection occurs after immunoreaction of immobilised PSA with Au nanoparticles conjugated with horse radish peroxidise and anti PSA antibody (adsorbed on gold nanoparticles). Further amplification of the signal is achieved by precipitation of the labelling enzyme with

DAB (3,3´-diaminodbenzidine) and H_2O_2 substrate solution (Cao *et al.*, 2007). On the other hand, these signal enhancement strategies make the immunosensor more complicated, increasing time of analysis, cost and decreasing the reproducibility.

5.4. Conclusions

In this chapter a low-cost, fast and simple SPR immunosensor for PSA detection in serum by employing a 3-D polymer for immobilisation of the antibody is described. Initially the possibility of using direct detection of PSA was studied. Thus different compounds (receptors such as BSA and antibody and blocking agents such as amino-PEG, ethanoloamine and aspartic acid) were immobilised on 3-D polymer and CM5 chips and the adsorption of 20% serum in PBS was measured. In the best case, the SPR response for serum adsorption on compounds immobilised on 3-D polymer was slightly over 1000 RU, which was still too high for useful clinical applications. On the other hand, on commercial CM5 chip very low serum adsorption was recorded after immobilisation of blocking agents, but not after immobilisation of antibodies. In this latter case a significant signal for adsorption of serum was recorded also on CM5 chips. Several methods were therefore applied in order to achieve reduction of serum adsorption such as inclusion of PEG in polymer composition and inclusion of a surfactant, 0.005% (v/v) of P20 both in running buffer and analyte solution. Whereas PEG 3-D polymer did not show enough improvement, a significant reduction of serum adsorption on polymer surfaces was observed using P20. Addition of the surfactant however did not reduce adsorption of serum on CM5 chips, where the antibody was immobilised. Nevertheless, despite the significant reduction of serum adsorption obtained on 3-D polymer chips using P20, the signal recorded in the best case was still too high (376.9 ± 91.4 RU) for clinical applications. Thus a sandwich

immunosensor was developed for PSA detection in serum. Firstly the sandwich immunosensor was optimised in PBS and a calibration curve of Biacore response *vs* PSA concentrations (ng ml^{-1}) was obtained. The detection limit of PSA in PBS was 0.1 ng ml^{-1} and the linear concentration range of the curve was between 1.0 – 250.0 ng ml^{-1}. Based on these results a detection of PSA in 20% serum solutions in PBS was performed. In this case the sensitivity of the immunosensor was lower and the detection limit was found to be 5.0 ng ml^{-1}. The application of 20% serum sample on Biacore is a novelty of our immunoassay compared with past publications reported in literature where 10% (v/v) serum was used (Cao *et al.*, 2006). Therefore our immunosensor is closer to successfully detect real samples despite the higher protein adsorption during sample injection. The deterioration of the sensor performace in serum compared with buffer detection, could be due to the adsorption of matrix components on the sensor, which caused restricted availability of the antigen epitope to the D-Ab paratope. The calibration curve of the sandwich sensor showed an earlier saturation compared with detection in PBS and the linear range for serum solutions spiked with PSA was from 5.0 to 100 ng ml^{-1}. The immunosensor developed here showed a detection limit in serum lower than many similar immunosensors reported in literature. Probably this is due to the 3-D structure of the novel polymer, which increases the availability of the antibody binding sites. Our immunosensor is therefore a step forward towards the development of a simple test, which could be successfully applied to detect PSA in real samples and therefore for early diagnosis of prostate cancer.

CHAPTER 6

CONCLUSIONS AND FUTURE WORK

6.1. General Conclusions

The current work aimed the development of new strategies for protein immobilisation on sensor surface. Initially SAMs produced by polythiol monomers were applied for kinetic studies and detection of cells by employing the reaction between primary amines and thioacetal groups, formed upon reaction of o-phthaldialdehyde (OPA) and thiol monomers. Initially the polythiol monomers ability to bind proteins in solution was assessed by recording the fluorescence upon reaction with OPA and NH_4OH. Once the polythiol SAMs were created they were characterised by contact angle and XPS. The possibility to immobilise proteins on monolayers was initially evaluated by employing BSA as a model protein. PETMP/OPA SAM showed overall the best performance and a general protocol suitable for the immobilisation of enzymes and antibodies such as anti-prostate specific antigen (anti-PSA) and anti *Salmonella typhimurium* was developed and optimised. Kinetic data were obtained for PSA binding to anti-PSA Ab and they were similar to the results obtained using Biacore commercial chips C1. For *Salmonella typhimurium* cells the PETMP SAMs chips exhibited better performance than C1. The detection limit was 5×10^6 cells ml^{-1} with a minimal non-specific binding. Despite these positive findings, it soon became clear that these polythiol SAMs were unable to bind enough receptors or to ensure high availability of receptors binding sites, requirements necessary for the achievement of highly sensitive sensors suitable for practical applications. Therefore polymers containing thioacetal groups, which allowed amine coupling without activation were also developed and applied on SPR sensors. The key step during polymers development was the inclusion of a 3-D crosslinker, which gave a tridimensional (3D)

network to the material and, ensured the availability of a high percentage of receptors binding sites. The resulting polymer layer was characterised using contact angle meter, atomic force microscopy (AFM) and ellipsometry. The resulting surface was relatively hydrophobic with an average contact angle of 67.2^{o}, calculated by measuring the interfacial tension of a drop of water on polymer-coated surface. The hydrophobicity of the surface was the main reason for the relatively high non specific binding of the material, especially when 3-D poymer chips were exposed to complex matrix like serum. The average thickness of the novel polymer layer was 5.3 nm, measured by using ellipsometry. This value is comparable to the thickness of the dry carboxydextran layer present on commercial CM5 chips. However, whereas the hydrophilic dextran layer can swell up to 100 nm in presence of aqueous solutions reaching high surface capacity the hydrophobic polymer developed in this work can not swell as much, resulting in a lower capacity. In order to assess the suitability of the 3-D polymer for development of SPR immunosensors, detection of PSA and microcystin-LR was performed by immobilising correspondent antibody on 3-D polymer chips. The results showed that indeed the novel 3-D polymer was suitable for development of immunosensors. In addition, it was found that the detection limits of the immunosensors for analytes were not only depended on the polymer, but also on the properties of antibody itself. The detection limit was 10 ng ml^{-1} for microcystin-LR and 0.05 ng ml^{-1} for PSA. The 3-D polymer chips also showed good stability as they were stored for up to 2 months without any noticeable deterioration in their ability to react with proteins. A significant advantage of 3-D polymer chips towards Biacore commercial CM5 chips was the possibility of immobilising any protein regardless of the chemical properties and charges. For example, a protein with a low pI such as pepsin was successfully immobilised on 3-D polymer chips, whereas its

immobilisation on CM5 was impossible. Other important advantages of the novel polymer, when compared with commercially available chips, are the low cost of raw materials necessary for its synthesis and the simplicity of the coupling reaction to bind biomolecules, which happens with a simple single step, without requiring any activation. On the contrary, immobilisation on CM5 requires an activation step performed with expensive (high purity) reagents (EDC/NHS).

After achieving successful detection of PSA in buffer solutions with a very low detection limit (0.05 ng ml^{-1}), the possibility of using the 3-D polymer to develop biosensors working in real samples, like serum was investigated. Detection of proteins in serum using the 3-D polymer resulted very challenging due to the complex nature of the matrix, which contains a high content of many different proteins. Direct detection of PSA in serum resulted to be impossible both with 3D- polymer and CM5 chips, due to the high adsorption of serum components on both surfaces. During this study several techniques were applied in order to reduce the non specific adsorption of serum on immobilised antibodies. These techniques include addition of polymerisable Polyethylene Glycol (PEG) in the polymer composition, blocking with a charged hydrophilic amino acid (aspartic acid) and amino-PEG and the inclusion of surfactants like P20 in the measuring system. Despite all these efforts, detection of PSA in serum was finally achieved using a sandwich format, where the analytical signal is recorded upon binding of a second antibody specific for a PSA epitope different from the one recognised by the antibody immobilised on the sensor surface.. The detection limits recorded with the sandwich immunosensors were 0.1 ng ml^{-1} in buffer and 5.0 ng ml^{-1} in serum, which is close to enable detection of PSA in serum samples without further amplification. Thus, this study has demonstrated the

opportunity to apply our novel polymer for development of sensitive and specific biosensors for biomarkers detection such as PSA in real samples.

6.2. Future work

Although the novel polymer developed in this work enable the development of SPR immunosensors for detection of PSA in serum, the following studies could be carried out to improve further polymer performances in complex matrices like serum.

- Optimisation of polymer composition in order to achieve higher capacity of sensor surface and consequently higher sensitivity in immunoassays by increasing the available binding sites.
- More hydrophilic polymer would be desirable since the layer could be swollen and the layer could be thicker and consequently we could achieve even higher availability of binding sites.
- A hydrophilic polymer also could result reduction of non specific binding and lower non specific adsorption of serum proteins making possible the direct detection of PSA in serum.

In addition to meliorate the polymer, improvement of sandwich assay sensors can be also obtained with the application of capture and detection antibody with very high affinity. This would be important for achieving even lower detection limits of biomarkers in serum and facilitate the use of biosensors for clinical applications.

Once all the polymer features mentioned above would be achieved, it could be a real possibility of mass production and commercialisation of our novel 3-D material for functionalisation of SPR and other optical and acoustic transducers.

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New reactive polymer for protein immobilisation on sensor surfaces

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ABSTRACT

Immobilisation of biorecognition elements on transducer surfaces is a key step in the development of biosensors. The immobilisation needs to be fast, cheap and most importantly should not affect the biorecognition activity of the immobilised receptor. A novel protocol for the covalent immobilisation of biomolecules containing primary amines using an inexpensive and simple polymer is presented. This tridimensional (3D) network leads to a random immobilisation of antibodies on the polymer and ensures the availability of a high percentage of antibody binding sites. The reactivity of the polymer is based on the reaction between primary amines and thioacetal groups included in the polymer network. These functional groups (thioacetal) do not need any further activation in order to react with proteins, making it attractive for sensor fabrication. The novel polymer also contains thiol derivative groups (disulphide groups or thioethers) that promote self-assembling on a metal transducer surface. For demonstration purposes the polymer was immobilised on Au Biacore chips. The resulting polymer layer was characterised using contact angle meter, atomic force microscopy (AFM) and ellipsometry. A general protocol suitable for the immobilisation of bovine serum albumin (BSA), enzymes and antibodies such as polyclonal anti-microcystin-LR antibody and monoclonal anti-prostate specific antigen (anti-PSA) antibody was then optimised. The affinity characteristics of developed immunosensors were investigated in reaction with microcystin-LR, and PSA. The calculated detection limit for analytes depended on the properties of antibodies. The detection limit for microcystin-LR was 10 ng mL^{-1} and for PSA 0.01 ng mL^{-1}. The non-specific binding of analytes to synthesised polymers was very low. The polymer-coated chips were stored for up to 2 months without any noticeable deterioration in their ability to react with proteins. These findings make this new polymer very promising for the development of low-cost, easy to prepare and sensitive biosensors.

1. Introduction

The market for immunoassay and immunosensor technology in areas such as food safety, drug discovery, environment and clinical analysis is set to grow, with a steadily increasing demand for cost-effective, sensitive and easy to use sensors (Sadana, 2006). One particularly important area of biosensors is the development of point-of-care and label-free devices, with special emphasis being dedicated to clinical assays for early cancer diagnostics (Sadana, 2006; Wang, 2006). In immunosensors or enzymatic sensors, proteins are either physically adsorbed onto the sensor surface (Predki, 2004) or covalently attached via amino or thiol groups (Kusnezow and Hoheisel, 2003). Common immobilisation methods include direct covalent attachment of receptors/ligands onto gold surfaces or the use of an intermediate matrix, such as polymers or self-assembled monolayer, to which the biomolecules are subsequently attached. Particular care is taken to protect the immobilised receptors/ligands from denaturing processes during or post immobilisation (Butler, 2000). In some cases attempts were made to achieve oriented immobilisation, wherein the receptor is attached to a surface by a particular part of the molecule (Vikholm, 2005; Cretich et al., 2006). It is believed that an oriented immobilisation provides superior orientation of binding sites as compared to a random immobilisation (Karyakin et al., 2000; Neubert et al., 2002; Kwon et al., 2004). Although beneficial in most cases, these processes include modification of the functional groups of the receptor molecule. Most of the techniques used are system-dependent and in some instances no actual improvement in binding performance was detected (Shriver-Lake et al., 1997; Kusnezow and Hoheisel, 2003). Other disadvantages of this oriented immobilisation include low and non-homogeneous protein deposition (Vijayendran and Leckband,

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2001), reduction of binding ability of the modified antibodies (Shriver-Lake et al., 1997) and expensive, time consuming manipulations of ligands (Shriver-Lake et al., 1997; Kusnezow and Hoheisel, 2003).

Another key requirement for a sensor surface is that the matrix used for the immobilisation of the receptor should produce a low level of non-specific interactions. This is because real samples such as serum are very complex and contain thousands of different molecules (Kusnezow and Hoheisel, 2003). A low level of non-specific binding is usually achieved by inclusion of polar molecules such as polysaccharides or polyethylene glycol derivatives into the immobilisation matrix (Masson et al., 2005). Concerning antibody immobilisation, it has been demonstrated in the past that flexible porous and non-porous polymer films, consisting of various molecular weight polymer fractions or different length spacer arms, perform better than homogeneous flat surfaces (Masson et al., 2005). This can be attributed to an improvement of protein diffusion in the polymer matrix, together with partial protection of protein structure from unfolding processes. Rigid or solid surfaces often cause irreversible denaturation of the bound proteins (Su et al., 1998).

Here we report the synthesis of a reactive polymer suitable for covalent immobilisation of proteins, or nucleic acids onto sensor surfaces. This novel polymer contains thiol derivatives (disulphide or thioethers) that promote self-assembling onto a metal transducer surface. The ligand immobilisation is based on the reaction between primary amines and thioacetal-containing polymer matrix (Simons and Johnson, 1978; Piletska et al., 2001). The product of the reaction is a fluorescence isoindole (Fig. 1a and b). This reaction can take place without any activation, which makes this novel polymer suitable for sensor/array fabrication. In addition the flexibility of the polymeric tri-dimensional (3D) network allows a high density of receptor immobilisation, while ensuring the availability of a high percentage of its binding sites. For demonstration purposes the 3D polymer was self-assembled onto gold Biacore chips and the entire testing was performed using the Biacore 3000. However there is potential to use this type of material on any noble metal transducer. Initially the ability of the polymer surface to bind proteins/receptors was tested by immobilising different ligands such as bovine serum albumin (BSA), three enzymes with different isoelectric points, one type of polyclonal antibodies (anti-microcystin-LR) and one type of monoclonal antibodies (anti-prostate specific antigen or anti-PSA). The results obtained for the immobilisation of BSA and the three enzymes on the polymer were compared with those obtained immobilising the same proteins on commercially available pre-functionalised Biacore chips (carboxydextran CM3 and CM5). Finally the quality of the receptor immobilisation was evaluated by binding the antigens (microcystin-LR and PSA) to the corresponding antibodies immobilised onto the novel polymer. The encouraging results observed during this work make this new polymeric matrix very promising for the development of low-cost, easy to prepare and sensitive biosensors.

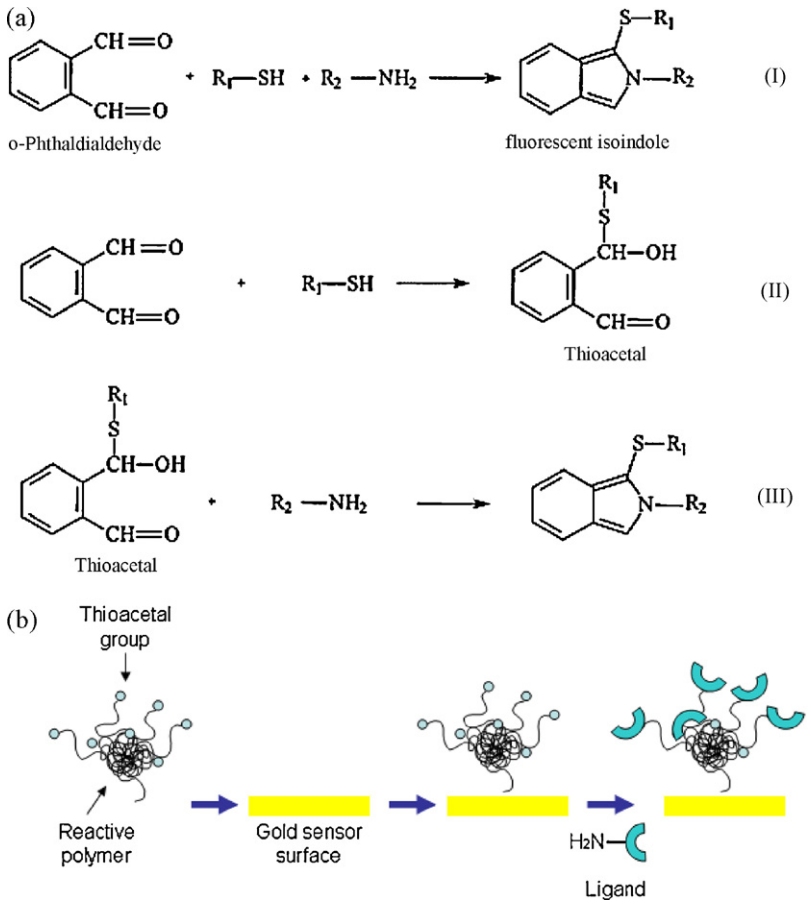

Fig. 1. (a) The reaction between *o*-phthaldialdehyde, mercaptan and primary amine (I), hemithioacetal formation (II), formation of the fluorescent isoindole complex between hemithioacetal and primary amine (III). R_1–SH is a polymerisable mercaptan, which will be included in the polymer, R_2–NH_2 represents any primary amine. (b) Schematic representation of biomolecule immobilisation on the tri-dimensional polymer-coated gold surface.

2. Materials and methods

2.1. Reagents

Most compounds were obtained from commercial distributors and were of analytical or HPLC grade. Triethylamine (TEA), bovine serum albumin (lyophilized powder), anti-sheep secondary antibody (anti-sheep Ab) were purchased from Sigma (UK). Sheep polyclonal anti-microcystin-LR antibody (anti-microcystin-LR Ab) was provided by Prof. Hennion from the Department of Environmental and Analytical Chemistry in Paris (Rivasseau and Hennion, 1999). Microcystin-LR was from Alexis (Switzerland). Monoclonal mouse anti-PSA antibody (anti-PSA Ab) and PSA were purchased from Serotec (UK) and Alpha Diagnostics (UK), respectively. Allyl thiol (AT), *N*,*N*′-bis(acryloyl)cystamine (BAC), *o*-phthaldialdehyde (OPA) were purchased from Fluka (UK). 2-Hydroxyethyl methacrylate (2-HEM), ethylene glycol dimethacrylate (EGDMA), 1,1,1-tris(hydroxymethyl)propan trimethacrylate (TRIM) and 2-benzyl-2(dimethylamino)-4′-morpholinobutyrophenone were purchased from Aldrich (UK). Ethanolamine, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), *N*-hydroxysuccinimide (NHS) and sodium dodecyl sulphate (SDS) solution were purchased from Biacore (Sweden). Solvents were of analytical or HPLC grade and supplied by Acros Organics (UK).

2.2. Polymer synthesis

The polymer was synthesised by mixing together 2.0 mmol (260 mg) of 2-HEM, 0.3 mmol (60 mg) of EGDMA, 1.5 mmol (507 mg) of TRIM, 1.0 mmol (134 mg) of OPA, 2.0 mmol (150 mg) of AT, 0.1 mmol (26 mg) of BAC, 0.5 mmol (180 mg) of 2-benzyl-2(dimethylamino)-4′-morpholinobutyrophenone (initiator) and DMF (5 mL) as solvent. A small amount of TEA (40 μL) was added to the monomer mixture and it was thoroughly purged with argon for 5 min. Polymerisation was initiated by placing the mixture under a high intensity Hönle 100 UV lamp (intensity 0.157 W cm^{-2}) for 20 min. The synthesised polymer was precipitated from DMF by adding 20 mL of water and washed several times with methanol.

2.3. Evaluation of polymer activity

The assessment of the polymer reactivity was carried out by measuring fluorescence produced by the isoindole deriving from the reaction of the thioacetal group of the polymer with primary amine groups. For the experiments 20 mg of the synthesised polymer were suspended in 3 mL of the following buffers: 0.1 M sodium phosphate buffer, pH 8.0; 0.01 M of phosphate buffered saline (PBS), pH 7.4; 0.1 M acetate buffer, pH 5.0 and pH 4.5. The emission of the suspension was measured between 400 nm and 460 nm in a 3 cm^3 quartz cuvette using a RF-5301 PC spectrofluorophotometer (Shidmatzu, Japan) with 370 nm as excitation wavelength. In order to detect the isoindole formation 5 μL of 6 M ammonium hydroxide were added to the polymer suspension and the emission recorded after 2 min incubation. The fluorescence maximum was recorded at 425 nm.

2.4. Polymer characterisation

The polymer fraction size was determined using GPC (gel permeation chromatography). Polymer was dissolved in tetrahydrofuran (THF) and 20 μL of 1 mg mL^{-1} solution were injected for analysis. The evaluation experiments were carried out using an Agilent 1100 SERIES HPLC system. HPLC analysis was performed at a flow rate of 1.0 mL min^{-1} with UV detection at 250 nm. The column was Phenogel 5 μm (Phenomenex®). The column was calibrated with polystyrene standards of 13–106 kDa purchased from Phenomenex®. The size of polymer particles was expressed in polystyrene equivalents and it was calculated from the calibration curve obtained by recording the retention times.

2.5. Polymer testing

Biacore 3000 (Sweden) and Au-coated chips (SIA Kit Au) purchased from Biacore (Sweden) were used in this work. All the experiments were performed at room temperature (25 °C).

2.6. Treatment of gold chips – gold surface modification

Sensor chips, SIA Kit Au (Biacore, Sweden) were used in order to assess the ability of polymer-coated surfaces to bind proteins. SIA Kit Au chips were cleaned for 3 min using oxygen plasma at 40 W in a plasma chamber (Emitech, UK). Polymer was self-assembled onto SIA Kit Au by immersing chips in 5 mL acetone/ethanol 50/50 (v/v) containing 10 mg mL^{-1} polymer for 24 h. The polymer-coated gold chips were rinsed thoroughly with acetone/ethanol, dried with nitrogen and assembled onto the holder.

2.7. Gold surface characterisation

The static contact angle measurements were made using a CCD camera supplied by Spectra Source Equipment model MCD400S (USA) with the software provided. The hydrophobicity was determined by measuring the interfacial tension of a drop of water on polymer-coated surface.

The thickness of polymer layer was defined by ellipsometry and atomic force microscopy (AFM). For the ellipsometry, the extinction coefficient of bare gold chips and the thickness values for polymer-coated sensors (seven sensor chips, three measurements per chip) were obtained using a SE 400 Ellipsometer (Mi-Net Technologies Ltd., UK). The roughness of the polymer layers was also measured using atomic force microscopy performed in contact mode using a PicoScan SPM from Molecular Imaging (USA).

2.8. Protein immobilisation on polymer-coated and carboxydextran surfaces

The protein immobilisation on sensor surfaces was monitored by Biacore 3000, which is a surface plasmon resonance (SPR) based instrument with a continuous flow system and four flow channels. The change in Biacore response units (RU) is directly proportional to the change of surface mass; 1 RU is approximately equivalent to 1 pg mm^{-2}. The biomolecules, which were used for studying the performance of the polymer-modified surface, were BSA, the enzymes trypsin, carbonic anhydrase and pepsin, sheep polyclonal anti-microcystin-LR Ab and mouse monoclonal anti-PSA Ab. Initially BSA was used as a model protein to assess the polymer binding capacity for biomolecules immobilisation. CM3 and CM5, which are Biacore carboxymethylated dextran chips used for biomolecule immobilisation, were tested for comparison. In this case, CM3 and CM5 were activated with EDC/NHS (Fagerstam et al., 1992) while polymer-coated surface did not require any activation stage. The activation of CM3 and CM5 was performed by injecting 30 μL of 0.2 M EDC/0.05 M NHS. Typically protein immobilisation was carried out on polymer-modified surfaces by injecting 100 μL of 100 μg mL^{-1} of protein solution in 0.01 M phosphate buffered saline, pH 7.4 with a flow rate 15 μL min^{-1}. Immobilisation of proteins onto CM3 and CM5 was performed in 0.1 M Na-acetate buffer, pH 5.0. The stability of immobilised biomolecules

on polymer-modified surfaces was tested by passing 5 μL of regeneration solution: 0.1%, SDS (sodium dodecyl sulphate) at a flow rate of 35 μL min^{-1}.

For the immobilisation of antibodies (anti-microcystin-LR and anti-PSA Ab) 100 μL (two injections of 50 μL) of antibodies solution (1/1000 dilution for anti-microcystin-LR and 2 μg mL^{-1} of anti-PSA) were injected in PBS with flow rate of 15 μL min^{-1}. After covalent coupling of the antibodies, remaining thioacetal groups were deactivated by injecting 50 μL of 0.2 M ethanolamine, flow rate 10 μL min^{-1}. The assessment of the reactivity of the immobilised antibodies was carried out by injecting corresponding antigens such as microcystin-LR and PSA. The antigens were diluted in PBS buffer and injected for 3–5 min, with a flow rate of 10 μL min^{-1}. In all experiments a reference channel containing polymer blocked with BSA was used for assessment of binding specificity. The surface was regenerated by injecting 5–10 μL of 10 mM HCl at a flow rate of 30–35 μL min^{-1}. The detection limit was calculated as the minimum analyte concentration, which produced a signal at least three times higher than the background noise.

3. Results and discussion

3.1. Polymer properties

o-Phthaldialdehyde and allylthiol in the presence of triethylamine react to create thioacetal groups, which can in turn react with primary amino groups with formation of a fluorescent isoindole. BAC was included in the polymer composition to supply disulphide groups useful for covalent attachment of the polymer on gold surfaces. 2-HEM increases the hydrophilicity of the polymer, usually associated with a reduction of non-specific protein adsorption. EGDMA is a bifunctional cross-linker and TRIM is a trifunctional cross-linker, which leads to a 3D polymer network and therefore to a larger surface area and higher availability of protein binding sites. In the absence of TRIM and with only EGDMA as cross-linker, the polymer capacity for proteins was up to 100 times lower. This was possibly due to the formation of a linear polymer, which produced a flat coating too closely attached to the gold surface and therefore unsuitable for sensor fabrication (results not shown). The main advantage of the 3D polymer lies in the opportunity to avoid binding site hindrance, which could be present in high density and flat antibody layers. Together with incorrect antibody orientation, this could lead to a high percentage of unavailable binding sites. The tri-dimensional network leads to a random immobilisation of antibodies further away from the sensor surface, but still in close proximity to allow the detection of antigen binding (see Fig. 1b).

When the reactivity of the synthesised polymer was assessed in solution at different pH values (by measuring the fluorescence of the formed isoindole group) a greater response was recorded in basic pHs. Nearly no fluorescence was observed for the polymer suspensions in acidic medium. The fluorescent intensity, which was recorded 2 min after addition of NH_4OH 6 M, was 615.7 ± 5.8% at pH 8.0; 512.3 ± 8.0% at pH 7.4; 64.4 ± 23.7% at pH 5.0 and 55.2 ± 33.8% for pH 4.5. The experiments were performed in triplicate. The fluorescence is a proof of the isoindole formation and therefore of the existence of thioacetal groups. The results also demonstrate the suitability of the polymer to perform protein immobilisation at physiological pH, which in most cases can be advantageous to avoid protein denaturation. The average molecular weight of the polymer, as determined by GPC was 110 kDa (polystyrene equivalent).

3.2. Characterisation of polymer-coated sensor surfaces

The polymer was attached to sensor gold surfaces by immersing the SPR chips in a polymer solution as explained in Section 2.6. The study of the obtained polymer layer included contact angle measurement ellipsometry and atomic force microscopy.

Contact angle measurements showed a moderately hydrophobic gold surface after modification with the polymer with an angle of 67.2 ± 6.0° (a very hydrophobic surface would have a value greater than 90°). The average thickness of the polymer coating was measured as 5.3 ± 1.1 nm using an ellipsometer. The layer is relatively thin if compared to Biacore chips CM3 and CM5, where the carboxydextran layer is 30 nm and 100 nm correspondingly. Thicker layers could be obtained by leaving the chips for longer time in the polymer solution (up to one week). This resulted not only in an increased capacity for protein, but also in an increased non-specific binding, so a thinner layer was considered optimal for further sensor work. The small deviation in the ellipsometry value shows that the polymer coating is homogeneous.

AFM measurements were carried out in contact mode for a further characterisation of the polymer layer. As it can be seen from Fig. 2a and b there is a change in the surface texture after the immobilisation of the polymer. A decrease in surface roughness was observed in presence of polymer (from 0.80 nm to 0.66 nm).

3.3. SPR experiments

The evaluation of the ability of polymer to bind proteins after attachment on a sensor surface was performed using Biacore 3000. BSA was employed as a model biomolecule and a protocol for protein immobilisation on polymer was optimised. Initially the attachment of BSA to the polymer-coated surface showed a satisfactory immobilisation level, which produced a Biacore signal of 1595 ± 3.2% RU with the experiments repeated 12 times. The immobilised BSA was washed with 10 μL 0.1% SDS solution (flow rate 35 μL min^{-1}) in order to assess the affinity of immobilisation. The material was relatively stable on the surface, and less than 5% of the immobilised BSA was removed from the sensor surface by the washing step. The BSA removed during the first washing step was most likely the one loosely adsorbed on the surface. The remaining BSA (covalently immobilised) was stable and no further material losses were detected.

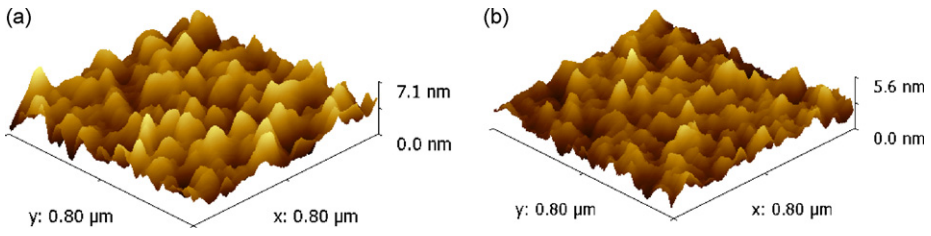

Fig. 2. AFM topographies of (a) cleaned gold substrate and (b) polymer-coated surface with respective roughness of 0.8 nm and 0.66 nm.

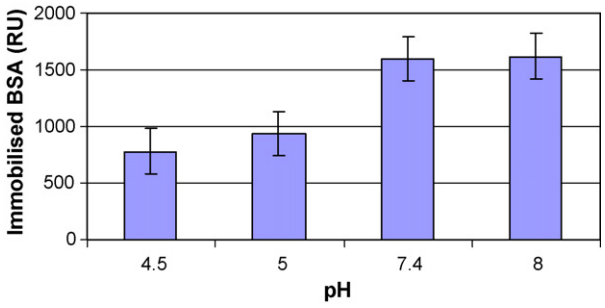

Fig. 3. Influence of buffer pH on the immobilisation of BSA (100 μg mL^{-1}) onto polymer-coated surfaces.

3.4. Effect of pH on BSA immobilisation

It is well known that protein immobilisation is highly dependent on pH (Branden and Tooze, 1999). In this work effect of a pH range of 4.5–8.0 (0.1 M acetate buffer pH 4.5, 0.1 M acetate buffer pH 5.0, 0.01 M PBS buffer pH 7.4 and 0.1 M phosphate buffer pH 8.0) on protein immobilisation was studied using BSA (100 μg mL^{-1}) as a model protein. The results are illustrated in Fig. 3. The experiment was carried out in triplicate. The result is consistent with the fluorescence experiments described above, where the lowest fluorescence was recorded at pH 5.0 and 4.5. The decrement of protein binding in acidic conditions could be due to an inhibition of the isoindole formation at low pH and also due to variations in protein charges induced by pH. Thus it is possible that more polar, negatively charged BSA at low pH will have lower affinity to relatively hydrophobic polymer surface. The highest immobilisation was achieved with PBS buffer pH 7.4 and 8.0. We believe that pH 7.4 would be ideal for protein immobilisation since it resembles physiological conditions.

3.5. Study of the bioreactivity of the immobilised antibodies

The maintenance of biorecognition activity of the ligands after immobilisation and minimisation of non-specific interactions between the sample and the recognition element are crucial for biosensor development. Thus, polyclonal anti-microcystin-LR Ab and monoclonal anti-PSA Ab were immobilised onto polymer-coated surfaces in order to study their ability to interact with their corresponding antigens microcystin-LR and PSA.

The first set of experiments was performed with anti-microcystin-LR Ab as the immobilised ligand and microcystin-LR as binding antigen. The immobilisation of antibody for a dilution of 1/1000 produced a Biacore signal of 2350 ± 11.7% RU calculated from 21 different experiments. The limit of detection for microcystin-LR was 10 ng mL^{-1}. This detection limit for the toxin is relatively high in comparison to that obtained when the same antibodies were first used (Chianella et al., 2002). This can be related both with the aging of the Ab, which was stored frozen for longer than 6 years, and most likely with the differences between the detection methods used in the two works: ELISA in the past *versus* direct SPR detection of a small analyte such as microcystin-LR in this work.

The sensogram showing the immobilisation of anti-microcystin-LR Ab and the immunoreaction is illustrated in Fig. 4. The figure also shows the regeneration step and the possibility to reuse the antibodies for further binding cycles. The amount of non-specific binding of analytes to the polymer was evaluated using a reference channel. In this channel the polymer layer was blocked with BSA (Kim et al., 2007) by injecting 100 μL of 100 μg mL^{-1} of the protein in PBS buffer, pH 7.4, followed by

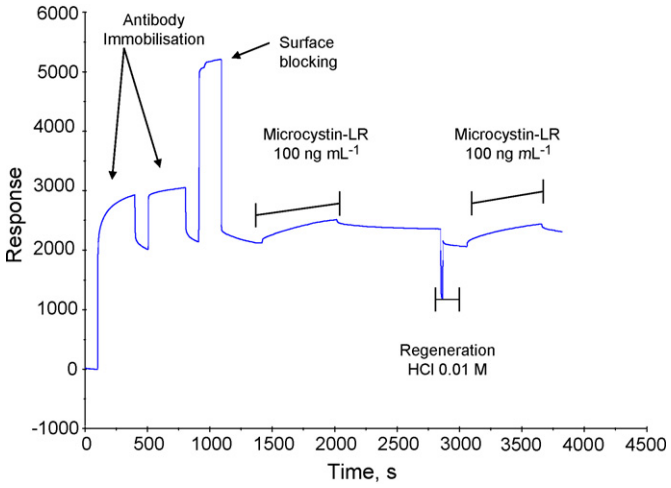

Fig. 4. A Biacore sensogram showing microcystin-LR immunodetection. The first two injections correspond to the antibody (polyclonal anti-microcystin-LR Ab) immobilisation; blocking of unreacted binding sites with 1 M ETA, pH 8.5 follows. The binding of 100 ng mL^{-1} microcystin-LR then occurs by a 50 μL injection in 0.01 M PBS buffer, pH 7.4 with a flow rate of 5 μL min^{-1}. Regeneration was performed by injecting 10 mM HCl for 10 s. At the end, a rebinding of microcystin-LR to regenerated Ab showing the same performance as in the first cycle is reported.

ethanolamine (ETA) blocking. The binding of the highest tested concentration of microcystin-LR (100 ng mL^{-1}) on this channel was negligible (lower than 20 RU). The low level of non-specific binding was also evidenced when, after immobilising a dilution 1/100 of anti-microcystin Ab (4618 ± 6.8% RU), anti-sheep Ab (specific for sheep anti-microcystin-LR Ab) was injected in the system and signals of 2456 ± 17.3% RU, and 534 ± 12.5% RU were obtained for working and reference (with polymer blocked by BSA) channels, respectively. The standard deviations were calculated for experiments performed in triplicate. These results also highlight the good availability of binding sites on the immobilised antibodies, with ca. 41% available, calculated considering that anti-microcystin-LR Ab and anti-sheep Ab have similar molecular weight.

In another set of experiments the binding of PSA to monoclonal anti-PSA Ab immobilised on polymer-coated chips was studied and the calibration curve, depicted on Fig. 5, was obtained after optimisation of the amount of antibody immobilised. The biosensor exhibited a linear detection range from 0.01 ng mL^{-1} to 1 ng mL^{-1} of PSA. This low limit of detection could be due to the usage

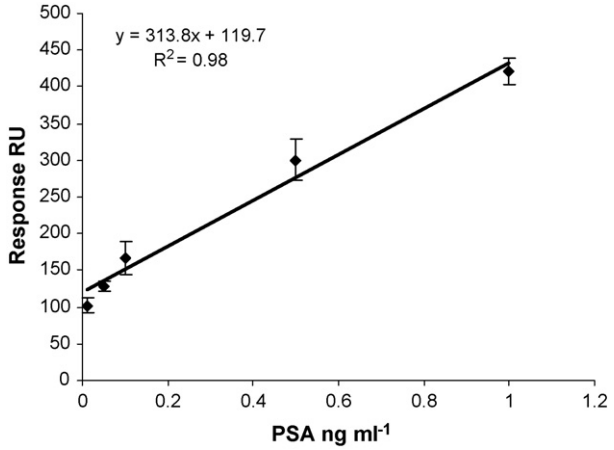

Fig. 5. Calibration curve of PSA obtained using monoclonal anti-PSA Ab immobilised on the polymer-coated surface.

Table 1
PSA binding in the concentration range of 0.01–1.0 ng mL^{-1} to monoclonal anti-PSA Ab immobilised onto the polymer-coated surface

PSA (ng mL^{-1})	PSA binding to monoclonal Ab (RU) (%)[a]	PSA binding to reference channel (RU) (%)[a]	Net binding signal (RU)
0.01	112.4 ± 8.8	10.3 ± 10.6	102.1
0.05	155.5 ± 4.5	26.4 ± 15.5	129.1
0.1	232.4 ± 9.9	65.1 ± 10.4	167.3
0.5	440.4 ± 6.7	140.0 ± 17.9	300.4
1	617.6 ± 2.9	197.1 ± 9.8	420.5

The non-specific binding to a reference channel blocked with BSA and ethanolamine is reported in the third column. Anti-PSA immobilised was 1976 ± 9.2% RU.
[a] Standard deviation was calculated from a set of three experiments.

of monoclonal antibody and the easy accessibility of the binding sites due to the structure of the polymer with approximately 80% binding site availability. The amount of immobilised anti-PSA was 1976 ± 9.2% RU and the net PSA binding was 420.5 ± 6.2% RU. The higher number of available binding sites when compared with the anti-microcystin/anti-(anti-microcystin) system described above might be explained with improved accessibility/diffusion of PSA due to a lower molecular weight (34 kDa for PSA and 150 kDa for IgG). The detection limit achieved in our system is lower than that reported elsewhere (0.027 ng mL^{-1}) obtained by enhanced SPR methods (Cao and Sim, 2007; Choi et al., 2008). Several concentrations of anti-PSA were tested (2.0 μg mL^{-1}, 1.0 μg mL^{-1}, 0.2 μg mL^{-1}, 0.1 μg mL^{-1}, 0.05 μg mL^{-1}, 0.02 μg mL^{-1}). The highest sensor responses were obtained with 2 μg mL^{-1} (1976 ± 9.2% RU), which also provided the lowest detection limit of PSA. This value was calculated for a set of 24 immobilisations and it demonstrates a good reproducibility, which could be further improved by automation of the polymer coating procedure. The storage of polymer-coated chips at room temperature and exposed to air for up to 2 months did not affect the reproducibility of these results. A longer stability study (usually 6 months) was not possible at the time.

For comparison purposes, the monoclonal anti-PSA Ab were also immobilised onto Biacore carboxymethylated dextran CM3 chip, using the immobilisation protocol suggested by the company. A very low antibody immobilisation was observed (302 RU). The quantity of the antibody immobilised on the chip surface was so low that it was difficult to detect the antigen PSA even at the highest concentrations. The calibration curve reported in Fig. 5 was obtained after subtraction of the non-specific binding, which was assessed by the amount of antigen binding to the reference channel (polymer channel blocked with BSA). The non-specific binding of PSA was increasing proportionally with PSA concentration; see Table 1.

3.6. Influence of pI on protein immobilisation

A challenging aspect during the development of sensors is the immobilisation of proteins with different isoelectric points (p*I*). For these experiments three enzymes (pepsin, carbonic anhydrase and trypsin) with p*I* ranging form 1 to 10 were tested. The results were then compared with those obtained with the same enzymes immobilised onto commercial Biacore chips CM3 and CM5. Both CM3 and CM5 are carboxymethylated dextran chips and biomolecules can be covalently attached to their surfaces by a peptide bond formation after activation of the carboxyl groups. The main difference between the two chips is the length of the carboxydextran matrix. The thickness of the dextran layer is 100 nm and 30 nm for CM5 and CM3, respectively, whereas the thickness of our polymer layer is around 5 nm as measured by ellipsometry. Therefore pepsin (p*I* = 1.0), carbonic anhydrase (p*I* = 6.5), trypsin (p*I* = 10) and BSA (p*I* = 4.5–5.5) were immobilised onto polymer-coated surface, CM3 and CM5. The results of the immobilisations are reported in Table 2. All the experiments were performed in triplicate.

As suggested by Biacore the immobilisation buffer used for CM3 and CM5 was 0.1 M acetate buffer, pH 5.0. At this pH a high negatively charged carboxydextran matrix is generated. Thus this charged matrix binds preferentially to positively charged proteins (proteins with p*I* higher than 5.0). Consequently all the proteins with a p*I* lower than 5.0 and therefore with a negative charge would not be able to easily approach the dextran layer. In addition at pH 5.0 some proteins might start denaturating. Hence the possibility of using a buffer with pH 7.4, which is very close to physiological conditions, for the immobilisation of ligands onto the polymer is a significant advantage. In fact Table 2 shows that successful immobilisation of pepsin (p*I* = 1.0) was achieved only on the polymer, whereas as expected practically no attachment was observed on CM3 and CM5, despite the significantly larger volume of CM3 and particularly CM5. As mentioned already this might have been caused by the electrostatic repulsion between the negatively charged carboxydextran matrix and the negatively charged pepsin. In contrast, the lack of a significant electrostatic charge on the polymer layer allows the negatively charged protein to approach the surface. The polymer-coated surface performed better than CM3 for most of the tested proteins with the exception of carbonic anhydrase. This might be due to the fact that this latter enzyme contains a zinc ion, which might promote electrostatic attraction to the negatively charged carboxydextran matrix.

CM5 showed in all cases, with exception of pepsin, higher binding capacity than the polymer-coated surfaces. Therefore polymer-coated surfaces are particularly advantageous when protein with low p*I* needs to be immobilised onto sensor surface. Another important advantage of the polymer-coated chips would be the cost. All the reagents involved on the synthesis of the poly-

Table 2
Immobilisation of enzymes and BSA on polymer-coated chip, CM3 and CM5 sensor chips

Protein	p*I*	Polymer-coated chip (RU) (%)	CM3 (RU) (%)	CM5 (RU) (%)
Pepsin	1	1165 ± 0.4	56 ± 21.4	39 ± 23.1
BSA	4.5–5.5	1596 ± 7.1	1232 ± 18.6	12030 ± 6.9
Carbonic Anhydrase	6.5	1780 ± 3.2	4450 ± 0.7	6845 ± 2.1
Trypsin	10	1716 ± 2.3	499 ± 12.2	4754 ± 7.4

The results were obtained using Biacore 3000.

mer are affordable, in contrast to CM3 and CM5, which are relatively expensive.

4. Conclusions

The work presented here describes the development of a new low-cost, fast and simple method for covalent immobilisation of proteins and other amino-containing biomolecules by employing an inexpensive and simple thioacetal-based polymer. This polymer offers a number of advantages. It contains groups that promote its self-assembling on a metal (preferable noble) transducer surface. It also contains groups that are able to bind amino-containing substances without any additional activation. Other desirable properties of the polymer are placement of appropriate amount of ligands and easy accessibility of binding sites given by the tridimensional polymer structure. Immunosensors developed on polymer-coated surface showed high sensitivity for the target analyte. In fact detection limits of 10 ng mL^{-1} and 0.01 ng mL^{-1} were obtained for microcystin-LR and PSA, respectively. Polymer-coated surfaces possess higher loading capacity for proteins with low p*I* when compared with commercial Biacore chips CM3 and CM5. All these advantages in combination with low non-specific binding, simple and inexpensive synthesis make this new polymer very promising for the development of low-cost, easy to prepare and sensitive biosensors.

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The application of polythiol molecules for protein immobilisation on sensor surfaces

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ABSTRACT

The immobilisation of bio-receptors on transducer surfaces is a key step in the development of biosensors. The immobilisation needs to be fast, cheap and most importantly should not affect the biorecognition activity of the immobilised receptor. The development of a protocol for biomolecule immobilisation onto a surface plasmon resonance (SPR) sensor surface using inexpensive polythiol compounds is presented here. The method used here is based on the reaction between primary amines and thioacetal groups, formed upon reaction of o-phthaldialdehyde (OPA) and thiol compounds. The self-assembled thiol monolayers were characterised using contact angle and XPS. The possibility to immobilise proteins on monolayers was assessed by employing BSA as a model protein. For the polythiol layers exhibiting the best performance, a general protocol was optimised suitable for the immobilisation of enzymes and antibodies such as anti-prostate specific antigen (anti-PSA) and anti *Salmonella typhimurium*. The kinetic data was obtained for PSA binding to anti-PSA and for *S. typhimurium* cells with a detection limit of 5×10^6 cells mL^{-1} with minimal non-specific binding of other biomolecules. These findings make this technique a very promising alternative for amine coupling compared to peptide bond formation. Additionally, it offers opportunity for immobilising proteins (even those with low isoelectric point) on neutral polythiol layers without any activation step.

1. Introduction

Immunoassay technology is currently growing rapidly due to market demands for low cost, easy to use and sensitive biosensors (Vikholm, 2005; Sadana, 2006). Surface plasmon resonance (SPR), quartz crystal microbalance (QCM), cantilever and electrochemical detectors are the most widespread platforms used with immunosensors. The main advantages of these when compared with immunoassays as ELISA, are the label free detection and the opportunity for measuring biochemical interactions in real time. This way kinetic and affinity constants can easily be obtained (Haga et al., 2008; Katsamba et al., 2006; Regnault et al., 1998).

The ligands (biomolecules) are usually attached on sensor surfaces by physical adsorption (Predki, 2004), covalent attachment (Kusnezow and Hoheisel, 2003; O'Shannessy et al., 1992) or ligand capture, which mainly refers to the strong interaction between biotilynated ligands and immobilised steptavidin or avidin (Craft et al., 1998; Panayotou et al., 1998). Covalent attachment is used because it provides a strong and stable binding of the ligand/receptor to the sensor surface. This allows easy regeneration of sensors using conditions which can remove the analyte from the surface, but not the attached ligand itself. Covalent immobilisation includes amino coupling (Lofas et al., 1995; Piletska et al., 2001), aldehyde coupling (Abraham et al., 1995) and thiol coupling methods (Johnson et al., 1995). The covalent attachment can also occur on gold surfaces modified with polymers such as carboxydextran matrix (Lofas et al., 1995) and thioacetal matrix (Kyprianou et al., 2009) or self-assembled monolayers (Nuzzo and Allara, 1983). The selection of the immobilisation procedure is a critical point for the development of a successful sensor. This is because the immobilisation may cause denaturation of ligand/receptor or alter the structure of binding sites (Butler, 2000) with consequent loss of bioreactivity. The direct attachment of the receptor on the sensor surface is however unadvisable since it can cause irreversible denaturation of the bound proteins (Su et al., 1998). The application of SAMs or polymers has advantages and disadvantages and selection of one over the other depends on the application. For example, flat surfaces with self-assembled monolayers (SAMs) are beneficial compared to polymeric layers (carboxydextran) both when the analytes of interest are large molecules such as cells and viruses

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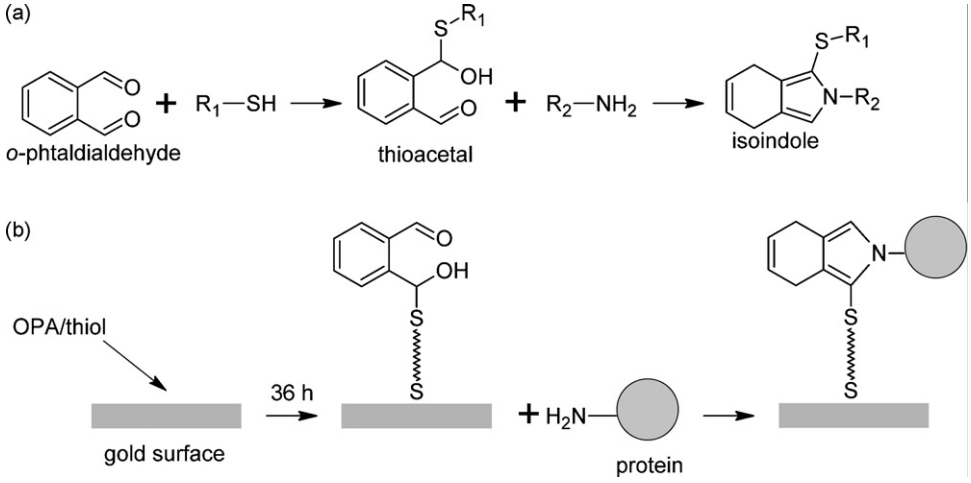

Fig. 1. (a) The reaction between o-phthaldialdehyde (OPA) and thiol with formation of hemithioacetal. Further reaction with primary amines will result in the formation of the fluorescent isoindole complex between the hemithioacetal and primary amine. R_1–SH represents a thiol molecule; R_2–NH_2 a primary amine. (b) Schematic representation of the process used for the immobilisation of proteins using the reaction described in (a).

and for kinetic parameters determination, when a low amount of non-specific binding is fundamental and low level of immobilised ligand is recommended (Biacore Sensor Surface Handbook). Gels matrices also complicate measurements due to diffusion restriction for large molecules and cells. The achievement of low or negligible nonspecific binding to sensor surface is another significant factor contributing to the success of sensor applications. Nonspecific binding contribution during measurement leads to positive standard errors in analyte determination and causes errors in calculation of kinetic constants, especially for complex sample matrices like serum (Kusnezow and Hoheisel, 2003). Reduction of nonspecific binding can be achieved by creating more hydrophilic sensor surfaces or by including compounds such as polysaccharides/polyethylene glycol derivatives in the immobilisation steps (Masson et al., 2005). Another way for reducing nonspecific binding is the addition of surfactants like P20 to analyte solutions (BIAapplications Handbook, 1994).

Here we report the development of SAMs on which the ligand immobilisation is based on the reaction between primary amines, thiol and o-phthaldialdehyde (OPA), see Fig. 1 (Simons and Johnson, 1978). This reaction takes place without any pre-activation of the surface making it suitable for sensor/array fabrication. In this study several thiols molecules were tested using both a spectrofluorophotometer (recording fluorescence upon isoindole formation at the end of the reaction) and surface plasmon resonance (SPR) with bovine serum albumin (BSA) as model protein. Among the thiols tested, the ones demonstrating the most promising results were applied for kinetic studies and analyte detection. The selected molecule which contains 4 thiol groups (pentaerythritol tetrakis (3-mercaptopropionate)) showed satisfactory protein binding and was unaffected during surface regeneration. In addition the SAMs obtained with this molecule showed stability and negligible nonspecific binding when tested by SPR. As a final study the results were compared to those obtained using corresponding commercially available sensors (Biacore chip, C1). Our novel monolayer proved to possess equal and in some cases improved features compared to the commercially available chips.

2. Materials and methods

2.1. Reagents

All compounds were obtained from commercial distributors and were of analytical or HPLC grade. Bovine serum albumin (lyophilized powder), IgG from bovine serum (95%), trimethylolpropane tris (2-mercaptoacetate) (TMPTMA), pentaerythritol tetrakis (3-mercaptopropionate) (PETMP), 1,6-hexanedithiol (HDT), 1,9-nonanedithiol (NDT), 2,5-dimercapto-1,3,4-thiadiazole (DMTZ) and the enzymes trypsin (lyophilised powder, from bovine pancreas), carbonic anhydrase (electrophoretically purified, dialised and lyophilised), pepsin (lyophilised powder from porcine gastric mucosa) were purchased from Sigma–Aldrich (UK). o-Phthaldialdehyde (OPA) and DL-dithiothreitol (DTT) were obtained by Fluka (UK). Mouse monoclonal antibody anti-PSA and anti *Salmonella typhimurium* (ST) as well as the native human prostate specific antigen (PSA) were purchased from Abd Serotec (UK). ST cells were kindly provided by Dr. Tothill research group (Cranfield Health, Cranfield University).

Ethanolamine, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-hydroxy-succinimide (NHS), sodium dodecyl sulphate (SDS) solution (0.5%, v/v), P20 (10%, v/v), NaOH solution (0.2 M), 10 mM glycine–HCl, pH 2.5, SIA Kit Au and C1 chips were purchased from Biacore (Sweden). Solvents were supplied by Acros Organics (UK). The water was purified by Milli-Q water system (Millipore, Bedford, MA, USA) and all the reagents used for Biacore experiments were filtered using a 0.22 μm teflon filter from Phenomenex®

2.2. Assessment of thiol reactivity

An initial assessment of the reactivity of different thiols was performed by measuring the fluorescence produced by the isoindole derived from the reaction between the thioacetal (after reaction with OPA) and primary amine groups (Fig. 1). Stock solutions were prepared by mixing thiols with OPA in molar ratio of thiol groups/OPA of 2:1 in DMF/ethanol (1:1) solution. Although all stock solutions contained 2.0 mM of thiol molecules the OPA concentration varied in order to maintain the molar ratio thiol groups/OPA 2:1. The resulting fluorescence was recorded every 15 min, after 1:10 dilution of stock solutions in DMF/ethanol (1:1) and addition of 7.5 μL NH_4OH 6 M as a source of primary amino groups. The emission of the solutions was measured between 400 and 460 nm in a 3 cm^3 quartz cuvette using a RF-5301 PC spectrofluorophotometer (Shimadzu, Japan) with 360 nm as excitation wavelength. Maximum fluorescence signal was observed between 430 and 440 nm.

2.3. SPR testing

The performance of different thiols on SPR sensor surfaces was evaluated by using Biacore 3000 (Sweden) and Au-coated chips (SIA

Kit Au) purchased from Biacore (Sweden). All the experiments were performed at 25 °C.

2.3.1. Treatment of gold chips–gold surface modification

Gold sensor chips, SIA Au (Biacore, Sweden) were used to assess the ability of polythiol/OPA monolayer to bind biomolecules. Au chips were cleaned for 3 min by oxygen plasma at 40 W using a plasma chamber (Emitech, UK). SAMs (except for DTT and DMTZ) were created on the gold surface by immersing the chips in 10 mL thiol/OPA solution in DMF/ethanol (1:1) with 2:1 molar ratio thiol groups/OPA for 36 h. Triethylamine TEA (50 μL) was added to the solution in order to facilitate thioacetal formation. The concentration was 0.1 M for di-thiol, 0.066 M for tri-thiol and 0.05 M for tetrathiol. OPA concentration was kept in all cases at 0.1 M. After immobilisation the gold surface was rinsed thoroughly with DMF/ethanol (1:1, HPLC grade), dried with nitrogen and the chips assembled on the holder.

For the water soluble dithiol molecules, DTT and DMTZ formation of SAMs was performed and recorded on-line using Biacore by injecting 200 μL on a cleaned gold chip (2 injections × 100 μL, flow rate 5 μL min^{-1}) of DTT/OPA (0.066 M/0.033 M) or DMTZ/OPA (0.02 M/0.01 M) prepared in 50 mM $Na_2B_4O_7$, pH 9.0. All the solutions were purged with argon for 5 min and kept under inert atmosphere in order to avoid oxidation. This experiment could not be performed with other thiols (insoluble in water) as Biacore is not compatible with organic solvents.

For the PETMP/OPA (the thiol with the best performance) the SAM formation was studied using film bulk acoustic resonators (FBAR) with gold electrodes. With a cell mounted on the FBAR which is open on the top it is possible to pipette amounts of solutions directly to the sensor gold surface. The technique is described in detail in Nirschl et al. (in press). To monitor the adsorption, the baseline was recorded with 10 μL of DMF/ethanol (1:1) in the cell. After a stable baseline was reached, 90 μL of PETMP/OPA monomer solution was added at a concentration of 0.1 M. The cell was then closed with a lid to avoid evaporation of the solution.

2.3.2. SAM coated sensor surface characterisation

The static water contact angle was determined with a CCD camera Supplied by Spectra Source Equipment model MCD400S (USA) with the software provided.

X-ray photoelectron spectroscopy (XPS) measurements were carried out on a VG ESCA lab-Mark-2 X-ray Photoelectron Spectrometer (East Grinstead, UK). The X-ray gun was operated at 14 kV and 20 mA. Survey and high-resolution spectra were collected at 50 and 100 eV respectively, with Mg Kα 1253.6 eV radiation. Scans were obtained in the C1s, N1s, O1s, and S2p regions of the spectrum. The decomposition of the XPS peaks into different components and the quantitative interpretation was performed after subtraction of the background using the Shirley method.

2.3.3. Protein immobilisation on coated gold surface

The biomolecules used for evaluation of performance of SAM coated surfaces were BSA (bovine serum albumin), the enzymes trypsin, carbonic anhydrase, pepsin and the antibodies mouse monoclonal anti-PSA and anti-ST. Non-immunoactive mouse IgG was used as control on reference channel for experiments with PSA and ST cells detection. BSA was used for the initial assessment of the capacity of SAM surfaces to immobilise protein. Biacore C1 chips were used for comparison. C1 was initially cleaned with 2 min injection (20 μL, flow rate 10 μL min^{-1}) of NaOH 1 mM containing 0.03% Triton X-100 the chip was then activated by injecting 70 μL (flow rate 10 μL min^{-1}) of 0.2 M EDC/0.05 M NHS (Fagerstam et al., 1992). Typically, BSA and enzymes immobilisation on SAM coated SIA Au was carried out by injecting 75 μL of 100 μg mL^{-1} of protein solution in 0.01 M phosphate buffered saline (PBS), pH 7.4 with a flow rate 15 μL min^{-1}. For the study of pH effect on protein immobilisation to thiol SAM, the proteins were diluted in buffer (0.05 M acetate buffer pH 4.5 and pH 5.0, 0.1 M PBS pH 7.4 and Na-borate buffer 0.05 M pH 9.0). For immobilisation of biomolecules on C1 0.05 M acetate buffer, pH 5.0 was used instead of PBS.

The stability of the immobilised biomolecules on SAM modified surfaces was tested by injection of 10 μL of regeneration solution: 0.1%, sodium dodecyl sulphate (SDS) at a flow rate of 30 μL min^{-1}.

For antibodies immobilisation (anti-ST anti-PSA and mouse IgG) 75 μL of antibodies (50 μg mL^{-1}) diluted in PBS pH 7.4 were injected with flow rate of 15 μL min^{-1}. Running buffer was also PBS, pH 7.4. For kinetic studies after antibodies immobilisation the buffer was switched from PBS to PBS containing 0.005% surfactant (P20) in order to eliminate nonspecific binding and improve fitting to the Langmuir 1:1 binding model. After covalent coupling of the antibodies, remaining thioacetal groups were deactivated with 25 μL 1 M ethanolamine hydrochloride at pH 8.5 and 2–4 injections BSA 30–50 μL (100 μg mL^{-1}). The evaluation of immobilised antibodies was performed by injecting the antigens PSA and ST cells into chip with corresponding antibodies. The antigens were diluted in PBS containing 0.005% of surfactant P20 and injected, for 3–5 min with a flow rate of 20 μL min^{-1} and 5 μL min^{-1} for PSA and cells correspondingly. PSA was injected at concentrations ranging from 3.3 to 832.5 nM. The dissociation time for assessing the dissociation constant K_d was 120–180 s. Kinetic data was obtained by Biaevaluation software provided by Biacore. In all experiments a reference channel with immobilised mouse IgG was used in order to assess the binding specificity. In case of anti-PSA/PSA the surface was regenerated with a pulse of 5–30 μL of HCl/glycine 10 mM (pH 2.5) at a flow rate of 30 μL min^{-1}. For the surface with immobilised anti-ST/ST cells, regeneration was performed by injection of 10–90 μL 1 mM NaOH, 30 μL min^{-1}.

3. Results and discussion

3.1. Reactivity of thiols

The ability of the selected molecules (DTT, PETMP, TMPTMA, DMTZ, HDT, and NDT) to form a fluorescent isoindole after reaction with OPA and NH_4OH was initially assessed for the selection of the most promising thiol molecules. Under the experimental conditions the maximum fluorescent was obtained at 3 h after NH_4OH addition. In higher molecule/OPA concentrations the maximum signal was reached 5–10 min after addition of NH_4OH. Nearly no fluorescence was observed for the molecule/OPA solutions in the absence of primary amines. The stability of the thioacetal groups (thiol/OPA solutions) was also studied by recording the fluorescence signal during 6 weeks at regular intervals after addition of NH_4OH. The fluorescent intensities, recorded 3 h after addition of NH_4OH for each molecule/OPA solution, are illustrated in Table 1. The experiments were performed in triplicate. Table 1 shows that the molecules exhibiting the highest fluorescence and therefore the strongest ability to form the isoindole and bind primary amine were DTT and PETMP. This can be explained by the presence of electron withdrawing groups (–OH for DTT and ester groups for PETMP) in the structures (See Fig. S1, Supporting Information) which increase the thiols acidity. As a result the thiol group is deprotonated more easily and the formation of thioacetal is facilitated. As expected, DTT showed limited stability because of its tendency to oxidise and decompose as the recorded fluorescence decreased considerably in 6 weeks. On the contrary, PETMP after 6 weeks in solution with OPA exhibited only a slight decrease in fluorescence. The fluorescence derived from the NDT/OPA and HDT/OPA reaction with NH_4OH was lower than the one obtained with the DTT and PETMP due to the lack of electron withdrawing groups.

Table 1
The fluorescence emission at 430–440 nm after reaction of thiol/OPA solutions with NH_4OH in DMF/ethanol 1:1.

Molecule	Maximum recorded fluorescence after NH_4OH addition						
	1st day	1st week	2nd week	3rd week	4th week	5th week	6th week
DTT	10.93 ± 3.1%	10.47 ± 4.3%	8.52 ± 6.3%	7.55 ± 2.1%	5.45 ± 4.4%	3.18 ± 6.3%	0.65 ± 9.2%
HDT	8.09 ± 2.7%	8.61 ± 3.3%	7.53 ± 4.2%	6.62 ± 5.3%	6.04 ± 5.2%	5.16 ± 3.4%	4.98 ± 3.3%
NDT	7.83 ± 5.1%	7.99 ± 4.2%	6.75 ± 2.9%	6.89 ± 3.1%	5.82 ± 3.5%	4.08 ± 2.8%	3.56 ± 5.9%
PETMP	10.11 ± 3.6%	9.93 ± 1.7%	9.40 ± 5.4%	9.40 ± 4.7%	9.58 ± 3.7%	9.31 ± 1.1%	9.14 ± 1.7%
DMTZ	–	–	–	–	–	–	–
TMPTMA		–	–	–	–	–	–

Table 2
Contact angle measurements of surface with polythiol molecules with and without OPA. The measurements were repeated three times.

	DTT	DMTZ	HDT	NDT	PETMP	TMPTMA
Contact angle thiol	16.3 ± 0.1	33.1 ± 0.6	55.0 ± 3.1	63.3 ± 0.1	51.6 ± 1.2	48.6 ± 1.3
Contact angle (thiol + OPA)	32.1 ± 1.5	63.4 ± 0.6	62.3 ± 0.1	63.3 ± 0.1	62.6 ± 0.7	53.1 ± 1.9

TMPTMA and DMTZ did not show significant fluorescence possibly due to the formation of an unstable isoindole, which can undergo decomposition quickly. Other possibility is the formation of non-fluorescent derivatives (Nakamura et al., 1982). Compounds with a long alkyl spacer between sulphur and oxygen and branching side-chains near thiol groups will yield isoindoles with increased stability (Jacobs et al., 1986; Stobaugh et al., 1983).

3.2. Characterisation of SAM coated sensor surfaces

SAMs were created on the gold surface by immersing the chips in thiol/OPA solutions. Immersing the gold chips into a polythiol solution without prior reaction with OPA can result in formation of a flat and disordered layer with restricted availability of thiol groups on the surface due to adsorption (on the gold) of more than one thiol group. In order to prevent this and produced 'well oriented' SAMs with thiol terminating groups, Niklewski et al. (2004) suggested the protection of one thiol group by creating a thioester and deprotection after SAM formation on sensor surface. Accordingly the presence of OPA in the solution plays a double role. It forms thioacetal groups, which are necessary for amino coupling and secondly, thanks to thioacetal formation, it prevents the flat orientation of SAM in the same way as thioesters do. The characterisation of all the resulting SAMs was performed by measuring the water contact angle as hydrophilic surfaces usually have lower unspecific protein adsorption. PETMP SAM, which exhibited the best performance in fluorescence experiments, was further characterised by XPS.

Contact angle measurement for all the SAMs with and without the presence of OPA is shown in Table 2, where it can be seen that DTT produces the most hydrophilic surface. The addition of OPA leads to thioacetal formation, which has a significant impact on surface as the contact angle increases from 16.3 to 32.1. DMTZ creates relatively hydrophilic surfaces, but the addition of OPA results in a hydrophobic SAM with contact angle of 63.4°. HDT, NDT, PETMP and TMPTMA form a relatively hydrophobic gold surface and the addition of OPA did not show significant influence on contact angle values.

XPS analysis revealed the presence of sulphur on the coated sample (6.2%) but not on the gold surface.

Monitoring the kinetics of the SAM formation on FBAR showed fast formation of a dissipative layer in the first few seconds which afterwards transforms into a less dissipative within a couple of minutes. Mass adsorption at saturation after 15 min was $31.6 \pm 4.5\,ng\,cm^{-2}$ which corresponds to 4.3×10^{13} molecules cm^{-2}, see Fig. S2 in Supplementary Data.

3.3. SPR experiments: assessment of SAM ability to immobilise protein

The ability of the SAM to immobilise proteins was studied using Biacore 3000. BSA was used as a model biomolecule. The experiment was repeated at least 3 times for each molecule and the affinity of immobilised BSA was assessed by injecting a solution of 0.1% SDS as described above. SDS can remove proteins loosely attached to the surface through hydrophobic interactions.

As illustrated in Table 3, DTT creates SAMs with high protein capacity. The standard deviation between DTT chips is high, probably due to the tendency of DTT to oxidise. This can influence the reproducibility of the results, since oxidation of thiols will lead to formation of disulfides which cannot form thioacetals. It is then crucial to keep the solution of DTT/OPA under inert atmosphere in order to prevent oxidation.

NDT and HDT are compounds with similar structure with the only difference being in the number of carbons atoms present in their carbon chains (see Fig. S1). Fluorescence results in solution have shown (see Table 1) that HDT performed better than NDT in producing isoindoles. However SAMs of NDT performed better than those of HDT in SPR tests. The protein capacity of the two SAMs is similar, but the stability of immobilised protein on the HDT SAM is significantly lower, since 26.4% of immobilised BSA was removed after SDS injection. This can be explained by the fact

Table 3
Biacore responses to BSA immobilised on different molecule SAMs. The standard deviation (STD) within the chip and between different chips is also reported. The last column shows material removed after injection SDS 0.1%.

Thiol	n[a]	Immobilised BSA (RU)	(%) STD within each chip	% Removed BSA after injection SDS 0.1%
DTT	6	1613 ± 21.4%	6.1	4.9 ± 0.4
HDT	3	1203 ± 7.7%	4.9	26.4 ± 3.7
DMTZ	3	1095 ± 14.1%	9.4	6.5 ± 1.3
NDT	3	1194 ± 24.2%	2.9	9.7 ± 1.6
PETMP	6	1487 ± 4.7%	2.2	4.3 ± 2.2
TMPTMA	4	1327 ± 10.0%	3.4	9.1 ± 5.5

[a] Number of chips used.

that a longer alkane chain (as on NDT) provides opportunity for more van der Waals interactions between neighboring molecules, allowing the formation of an ordered thiol layer (Bain et al., 1989; Holmes-Farley et al., 1988). HDT most likely produces a SAM, which is not as ordered.

TMPTMA and PETMP are molecules containing three and four thiol groups respectively. PETMP SAMs showed higher surface capacity for protein immobilisation with 1487 RU and TMPTMA layer 1327 RU. Additionally, as illustrated in Table 3 PETMP layer exhibited higher stability as only 4.3% of the immobilised BSA was removed after washing with SDS, whereas 9.1% was removed from TMPTMA layer. The high stability of this molecule was also proven by the low STD (4.7%) for protein immobilisation calculated using six different chips over a 3 months period. On the contrary TMPTMA seemed to be affected by stability problems especially after exposure to atmospheric conditions. Despite the fact that TMPTMA and DMTZ did not show fluorescence in solution the observed protein immobilisation can be due to other reactions of thioacetals with amino groups, which results in non-fluorescent products (Nakamura et al., 1982).

It can be concluded that SPR experiments are generally in agreement with fluorescence studies (Section 3.1). Both have shown that the molecules with highest performance for amine coupling are DTT and PETMP. The fluorescence experiments have shown PETMP as one of the most stable molecule and DTT as one of the least stable. Due to its high reproducibility, stability and good affinity PETMP was selected for further studies.

3.4. Application of PETMP/OPA SAM and comparison with Biacore chip C1

3.4.1. Kinetic analysis of PSA/anti-PSA monoclonal antibody interaction

Flat sensor surface modifications are useful especially for the determination of kinetic constants and evaluation of affinity of binding reactions. Application of flat surfaces with low volume and restricted surface capacity is important due to the fact that in this condition mass transport limitation has a minor impact on the resulting sensogram (Önell and Andersson, 2005). In order to minimise the limitation of mass transport when a sensor chip with high surface capacity is applied for kinetic studies the immobilisation level of the ligand should be kept low (500–2000 RU) (Katsamba et al., 2006). Functionalisation providing low volume surfaces can also result in less nonspecific binding (due to limited charge attractions and hydrophobic interactions), which, if it is not eliminated, can have a prominent effect on the calculation of kinetic constants. Biacore also recommends low capacity sensor surfaces for kinetic studies (Biaevaluation Handbook).

The effect of the pH on protein immobilisation on PETMP/OPA SAM was studied in the range 4.5–9.0 (0.05 M acetate buffer pH 4.5, 0.05 M acetate buffer pH 5.0, 0.1 M PBS pH 7.4 and 0.05 M borate buffer pH 9.0). The highest immobilisation was achieved when BSA was immobilised in PBS buffer pH 7.4 and borate buffer pH 9.0 as expected since the fluorescent isoindole formation is inhibited in acidic conditions. Consequently the immobilisation at pH 7.4 was selected for further studies on PETMP/OPA SAM since it resembles physiological conditions while still allowing for good protein immobilisation.

Comparative studies between PETMP/OPA SAMs and Biacore C1 sensor chips were performed with monoclonal anti-PSA. Immobilisation of antibody on PETMP-OPA and C1 produced a signal of $4572 \pm 2.3\%$ and $3145 \pm 2.7\%$ resonance units (RU) respectively. Each experiment was performed in triplicate. The kinetic constants, determined with the Biaevaluation software, provided by Biacore, were k_a (the rate of formation of new complexes), k_d (the rate of complex dissociation) and K_D (equilibrium dissociation constant).

Table 4
The calculated kinetic constants for anti-PSA/PSA interaction on C1 and on PETMP-OPA SAM.

	k_a (10^4)	$T(k_a)$	k_d (10^{-3})	$T(k_d)$	K_D (nM)	x^2
PETMP-OPA	4.52	35.4	4.77	10.2	106	1.54
Biacore C1	5.36	49.1	4.19	18.1	79.3	0.341

In general for a 1:1 Langmuir binding model, kinetic constants values between 10^4 to 10^7 for k_a and 10^{-4} to 10^{-1} for k_d can be determined with high confidence. Higher or lower kinetic constants might be affected by errors produced either during experiments or during the fitting of the data (Önell and Andersson, 2005). In this study PSA in varying concentrations (3.33–166.5 nM for PETMP/OPA SAMs and 33.3–832.5 nM for C1) were injected on both surfaces for each fitting. The concentrations of PSA used on C1 surface were higher because, due to lower surface affinity, low concentrations resulted in inadequate responses. Very low concentrations, as well as high concentrations were avoided in the attempt to obtain more precise fittings. In fact at low concentration level the noise contribution may be significant and at high concentrations other interactions can lead to deviations from the simple 1:1 Langmuir model. The sensograms obtained with both sensor chips fitted well the 1:1 Langmuir binding model (see Fig. S3 in Supplementary Data). The parameter x^2, which is used to measure the accuracy of the fittings, had a value of less than 2 which indicates excellent fitting (Biaevaluation Handbook). Specifically the x^2 values for PETMP-OPA and C1 sensor were 0.341 and 1.54 respectively (Table 4). Statistical information on the data is given by the *T*-value, which is the relative measure of the standard error. *T*-value is determined by dividing the value of the parameter (in this case k_a and k_d) by the standard error. A *T*-value higher than 10, which corresponds to less than 10% standard error, is considered satisfactory (Biaevaluation Handbook; Önell and Andersson, 2005).

The values of K_D obtained here (Table 4) demonstrate relatively low affinity for PSA antigen if compared to previous studies where different monoclonal Ab showed much higher affinity with a K_D of only 1 nM (Katsamba et al., 2006). The differences of the association constant k_a and dissociation constant k_d values calculated for the two surfaces for the same antibody–antigen reaction are satisfactory since deviations for kinetic constants determination of 15–20% are acceptable (Myszka et al., 1998; Katsamba et al., 2006; Önell and Andersson, 2005). The similarity between kinetic values obtained on PETMP-OPA and C1 surfaces shows that both can be used as a low capacity flat surface sensor for kinetic studies.

3.4.2. Detection of Salmonella typhimurium cells

Other important application for flat surfaces in SPR is the detection of large analytes like cells or virus. The reason is that, since the evanescent wave for SPR measurements is only 300 nm from the sensor surface, the use of chips as CM5 with high capacity layers of around 100 nm, makes detection of large molecules challenging. In fact, large analytes have shown poor responses on such surfaces as the sensitivity decreases exponentially with the distance from the sensor surface (Biacore Sensor Surface Handbook; Nagata and Handa, 2000). Another issue is that because of their dimensions, only a restricted amount of large analytes can be immobilised on the surface. Hence the newly developed SAM and C1 were both applied for detection of ST cells and the results compared. For this application anti-ST antibodies were immobilised both on PETMP-OPA SAM and C1 chips. The antibodies immobilisations produced Biacore signals of $2461 \text{ RU} \pm 2.9\%$ and $1543 \text{ RU} \pm 5.7\%$ for PETMP-OPA and C1 respectively. Standard deviations were calculated using three separate immobilisations. The newly developed surface showed higher capacity than C1 for antibodies immobilisation. After blocking with TEA and BSA, several cells dilutions

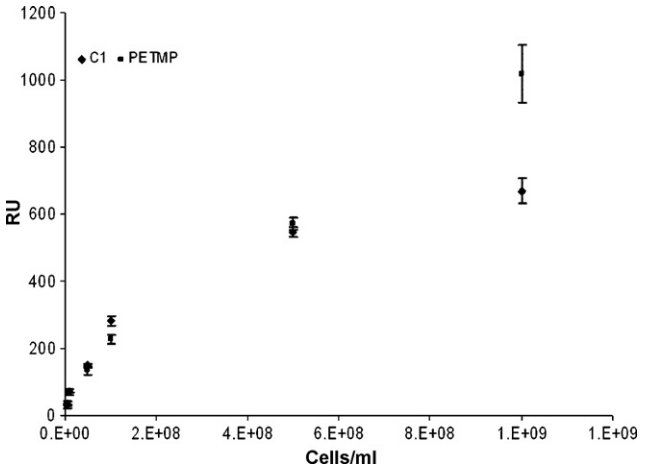

Fig. 2. Calibration curve for ST cells detection obtained using monoclonal anti-ST antibody immobilised on PTEMP-OPA and C1 surface.

prepared in PBS buffer were injected both on working and reference channel with a reduced flow rate (5 μL min^{-1}) in order to avoid blockage of the injection system. The detection of cells in real time is illustrated in Fig. S4 of the Supporting Information. The resulting calibration curves are reported in Fig. 2. The curves were calculated by subtracting the response recorded on the reference channel for each concentration. The addition of surfactant improved the performance of the immunoassay due to the reduction of unspecific binding (ca. 3 times reduction of the recorded response on the control channel). The nonspecific binding was in general higher for the PETMP SAMs especially for the two highest cells concentrations but never exceeded 10%. Nonspecific binding was negligible for C1 even without surfactant. The higher amount of nonspecific binding to PETMP SAM is possibly due to the hydrophobicity of the surface.

Due to higher amount of antibody immobilised on the PETMP surface, this chip exhibits higher capacity when high concentrations of cells are used (Fig. S4). The lowest detectable concentration with at least three times higher response than that of reference channel was 5×10^6 cells mL^{-1} for PETMP SAM and C1 sensor chip. The sensitivity and consequently the detection limit for immunoassays are highly dependent on the affinity of the applied antibody. A lower detection limit (1.25×10^5 cells mL^{-1}) for the same bacteria was for example reported by Mazumdar et al. (2006) using a sandwich assay. The highest sensitivity could be due both to the quality of the antibody used by or by the detection system (sandwich versus direct assay).

3.5. Study of pI effect for proteins immobilisation on PETMP SAM and C1

A challenging aspect during sensor fabrication is the immobilisation of proteins with different isoelectric points (p*I*) on the same substrate. For these experiments proteins with p*I* ranging from 1 to 10 were tested on PETMP and also on C1 for comparison. Pepsin (p*I* 1.0), carbonic anhydrase (p*I* 6.5), trypsin (p*I* 10) and BSA (p*I* 4.5–5.5) were immobilised on the chips and the response monitored on Biacore. As suggested by Biacore, the pH of the buffer used for immobilisation on C1 chips was 5.0, as this produces maximum attachment of positively charged protein to the negatively charged carboxylic group present on the sensor surface. An acidic pH is in fact required to immobilise positively charged proteins. The use of a pH lower than 5.0 is however not advisable as it might inhibit peptide bond formation. This means that proteins with a p*I* lower than 5.0–5.5 will be difficult to attach to the C1 surface. Table S1 of Supporting Information shows that the capacity of the novel SAM for all the tested proteins. The most significant advantage as shown is the possibility to immobilise pepsin, which is a protein with a very low p*I* (p*I* = 1) onto PETMP SAM, with nearly no attachment onto the C1, with 1102.4 RU on PETMP and 46.1 RU on C1. This is most likely due the electrostatic repulsion between the negative charges present on the protein at pH 5.0 and the negatively charged carboxylic group present on C1 layer. On the other hand, the application of amino coupling SAM produces a neutral surface which lacks significant electrostatic charges. Hence, negatively charged proteins can approach the surface in close proximity and immobilise easily onto the SAMs. Another advantage resulting from the absence of charges on the developed PETMP SAM surface is the opportunity to detect charged analytes by avoiding the non-specific binding caused by electrostatic attractions of interfering compounds with similar charges. In conclusion the novel polythiol SAMs reported here seems to be generic reagents capable of immobilising proteins regardless of their chemical properties such as p*I*.

4. Conclusions

The work presented here describes the development of a novel, low cost, fast and simple method for polythiol SAMs formation on gold surface, which enables immobilisation of protein through amino coupling using a chemistry which is an alternative to peptide bond formation. The amino coupling is based on the reaction of thioacetals, formed by reaction of thiols groups with aldehydes, with primary amino groups resulting into the formation of fluorescent isoindoles. On the contrary to many methods used for amino coupling, the thioacetal groups are able to bind amino containing substances without any pre-activation step. Several thiol-containing molecules were tested for their ability to form thioacetal self-assembled monolayers (SAMs) and their capability to immobilise protein assessed. Among those tested, PETMP SAMs showed the highest stability and surface capacity for proteins immobilisation. PETMP SAMs was utilised for kinetic studies of anti-PSA–PSA interaction and for the detection of ST cells. The performance of the novel SAM was then compared with the commercially available Biacore C1 chips. Both in kinetic study and in the detection of cell the results obtained with PETMP SAMs were comparable with those achieved by using the C1 chips. Kinetic constants determined for anti-PSA/PSA were $k_a = 4.52 \times 10^4$, $k_d = 4.77 \times 10^{-3}$ and $k_a = 5.36 \times 10^4$, $k_d = 4.19 \times 10^{-3}$ for PETMP SAM and C1 correspondingly. ST cells were detected down to 5×10^6 cells mL^{-1} with both surface-modified chips. Another advantage of PETMP SAM is its ability to immobilise proteins regardless of the isoelectric points. Additional benefits would come from the low cost involved in polythiol SAM formation and the simplicity in using generic protocol required for immobilisation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2009.09.030.

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