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Y ULUDAĞ

**DEVELOPMENT OF AN AFFINITY SENSOR FOR
PROSTATE CANCER DIAGNOSIS**

CRANFIELD HEALTH

PhD THESIS

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Yıldız Uludağ

**Development of an Affinity Sensor for Prostate
Cancer Diagnosis**

Supervisor: Dr. Ibtisam E. Tothill

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ABSTRACT

Prostate carcinoma is a fatal malignancy and is a major cause of death in men in the population aged 55 and over. Early diagnosis of prostate cancer is very important for successful treatment of the disease. The increase in prostate specific antigen (PSA) levels in serum above the normal limits is the primary indication of prostate malignancy; therefore, PSA is used as a biomarker for the diagnosis and prognosis of prostate cancer. To date PSA testing still dominates as the best biomarker for prostate cancer detection even though it is not prostate specific. This project aims to develop a rapid, sensitive and specific biosensor to detect prostate cancer biomarkers in order to reduce the number of people who undergo invasive examinations for diagnosis.

As part of the work, alternative surface chemistry techniques were investigated to optimise the sensors immobilisation capacity, such as the use of dendrimer monolayers and surface grafting polymers. It was found that dendrimer monolayers can be used to increase the sensor surface capacity by 1.5 times for surface plasmon resonance (SPR) sensor chips but not for quartz crystal microbalance (QCM) chips. A novel polymer (UVlink) surface was also fabricated and successfully used for biomolecule immobilisation for SPR and QCM chips. The QCMA-1 prototype biosensor was applied to develop PSA detection assays. Initially an immunoassay was constructed and optimised on the QCM sensor chip. A new buffer was then formulated to eliminate 98% of non-specific binding due to human sera proteins and this enabled the PSA detection assay to be performed in 75% human serum. Since QCMA-1 instrument is a prototype, to confirm the results the PSA assay was repeated using a commercial biosensor, Biacore 3000. This newly developed method showed a limit of detection of 0.29 ng mL^{-1} (in 75 % serum) with a linear dynamic

detection range up to 150 ng mL^{-1} with Au nanoparticles employed for sensitivity enhancement using both instruments. With the achieved detection limit, it is possible to use the developed QCM and SPR assays for cancer detection in patient samples. PSA detection binding data fitted to 1:1 Langmuir binding model and K_D calculated as $9.46 \times 10^{-10} \text{ M}$ for the assay performed with Biacore 3000 instrument and using the QCMA-1 biosensor $5.56 \times 10^{-10} \text{ M}$. These results were comparable and show that the affinity between PSA and PSA-capture antibody is in the region of 10^{-10} M and that QCMA-1 assay results are compatible with Biacore 3000 assay results. In the study incorporation of fPSA, cPSA and PSMA immunoassays to the prostate cancer detection test was also considered. However, the commercial available antibodies were formed to have low affinity for these markers. Patient samples collected from Bedford Hospital NHS Trust and control samples collected from Cranfield University staff were tested and it was found that the developed QCMA-1 test clearly discriminates the PSA level of patients from the controls.

A second biosensor platform based on label-free interdigitated capacitive sensing principle was also employed in the study. Interdigitated electrode (IDE) arrays were fabricated with conventional microelectronics-micromachining technologies on silicon wafers. Later IDE arrays were utilised for the detection of PSA using an LCR meter. This new prototype enabled capacitance reading of individual capacitors in turn and also enabled the detection to be performed in buffer solution. Antigen binding assays were developed to capture the PSA molecule in buffer solutions achieving a detection limit of 15.6 ng mL^{-1} with a linear dynamic detection range of $15.6 - 250 \text{ ng mL}^{-1}$ PSA.

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LIST OF ABBREVIATIONS

AGP - Human alpha-1 acid glycoprotein
ACT - α -1-antichymotrypsin
Amol - Attomol
Au - Gold
BAW - Bulk Acoustic Wave
BPH - Benign prostatic hyperplasia
BSA - Bovine serum albumin
CMD - Carboxymethyl dextran
DMF - Dimethyl formamide
DNA - Deoxyribonucleic acid
DRE - Digital rectal examination
EDC - 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide
EDMA - Ethylene glycol dimethacrylate
ELISA - Enzyme-Linked ImmunoSorbent Assay
EPCA- Early Prostate Cancer Antigen
EU – European Union
fPSA - Free PSA
FDA - Food and Drug Administration
GSTP1 - Glutathione S transferase P1 gene
hK - Human glandular
HRP - Horse radish peroxidase
IGFs - Insulin-like growth factors
IGFBPs - Insulin-like growth factor binding protein
KCl - Potassium chloride
M - Molar
mM - Millimolar
MA - Methacrylic acid
MCM - Mini-chromosomal maintenance
MEA – Microelectrode array
MEMS - Microelectromechanical systems
MUDA - Mercaptoundecanoic acid
mg - Milligram
ml - Millilitre
mRNA - Messenger RNA
 μ m - Micro meter
NEA - Nanoelectrode array

ng - Nanogram
 NHS - N-hydroxysuccinimide
 nM - Nanomolar
 NMP⁺ - N-methylphenazin-5-ium cation
 PAMAM - polyamidoamine
 PAP - Prostatic acid phosphatase
 PBS - phosphate buffer saline
 PCA3 - Prostate Cancer gene 3
 PCB - printed circuit board
 PCR - Polymerase chain reaction
 PIN - Prostatic Intraepithelial Neoplasm
 pM - picomolar
 PSA - Prostate specific antigen
 PSM - Prostate-specific membrane antigen
 QCM - Quartz crystal microbalance
 RCA - The plant rection ricin
 RNA - Ribonucleic acid
 Rms - root mean square
 Rpm – revolutions per minute
 RT-PCR - Reverse transcription polymerase chain reaction
 SAM - Self assembled monolayer
 SEM - Scanning electron microscope
 SPE - Screen printed electrodes
 SPR - Surface plasmon resonance
 TCNQ - Tetracyanoquinodimethane
 TMB - 3,3',5,5'-tetramethylbenzidine hydrochloride
 tPSA - Total PSA
 TRUS - Trans-rectal ultrasound guided biopsy
 TSM - Thickness-shear mode
 TTF - Tetrathiafulvalene
 UK - United Kingdom
 UME – Ultramicroelectrode
 USA - United States of America
 WHO - World Health Organisation

CHAPTER I:
INTRODUCTION AND LITERATURE REVIEW

1 INTRODUCTION AND LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

Prostate cancer, the commonest form of cancer in men, is a complex, multifactorial disease. Early and accurate detection of prostate cancer is very important, since there is no cure if the cancer spreads out to the other organs of the body. This makes early diagnosis very important for successful treatment of the disease.

The increase in prostate specific antigen (PSA) levels in serum above the normal limits (4 ng mL^{-1}) is the primary indication of prostate malignancy; therefore PSA is used as a biomarker for the diagnosis and prognosis of the prostate cancer (Brawer and Lange, 1989; Diamandis, 1998). The current PSA tests rely on ELISA type assays and usually performed at centralised laboratories using expensive automated analyzers and time consuming procedures (Healy et al., 2007). This process requires sample transportation, increased waiting time and high medical costs. Therefore, there is a need to develop a detection system that is cheap, quick to process, uses low sample and reagent volume and easily operated. Additionally due to the complexity of prostate cancer, there is an increasing need to test multiple biomarkers simultaneously for cancer diagnosis/prognosis. Recent advances in the area of sensor technology and microarrays have enabled the miniaturisation of the devices and multiplex testing of a range of analytes. Therefore, biosensor technology has the potential for the manufacture of point-of-care cancer testing devices.

In the current study, for prostate cancer detection, both quartz crystal microbalance (QCM) and electrochemical biosensors have been considered. These include two prototypes developed by two collaborators Sierra Sensors GmbH (Germany) and Sabanci University (Turkey). Ultimately these biosensors will be used to further develop to detect multiple biomarkers in serum for the rapid and accurate diagnosis of cancer that will not require invasive examinations.

Sierra Sensors GmbH recently launched the first prototype of QCMA-1 biosensor. The advantages of this biosensor include label-free real time biomolecular interaction monitoring, rapid assay that uses small sample size, repeated binding and regeneration can be performed on the same sensor chip and flexible experimental design. A collaboration study with Sierra Sensors GmbH has been started to test of QCMA-1 prototype and compare the results with a commercial biosensor instrument (Biacore 3000). Additionally new surface chemistries have been developed to increase the sensitivity and dynamic range of the QCMA-1 assays and later this instrument was used for PSA detection in patient samples collected from Bedford Hospital.

As an addition to use of a QCM biosensor for prostate cancer detection, electrochemical biosensors are considered. Collaborators at Sabanci University (Turkey) develop a biosensor using interdigitated electrode arrays. The biosensor developed by Sabanci University has been tested and contributions have been made for the further development of the system.

Here in the introduction chapter of this thesis, the results of the literature survey is discussed. First information is given about prostate cancer and biomarkers with a particular emphasis on the prostate specific antigen (PSA). Then, different types of biosensors are summarised as potential diagnostic tools. In the last section examples of biomarker detection assays using biosensors are given.

1.2 PROSTATE CANCER DIAGNOSIS

1.2.1 Prostate cancer

Cancer is a general term for a set of diseases that are characterised by abnormal and uncontrolled growth of body cells. Cancerous cells can travel through the body by means of bloodstream or lymphatic system and by this way spread to the other parts of the body. According to the World Health Organisation (WHO) around 13% of all deaths worldwide were caused by cancer in 2007 that accounts for 7.9 million. It is predicted that by 2030 the deaths from cancer will increase up to 12 million (WHO, 2008). Among different cancer types, prostate cancer, the commonest form of cancer in men in Europe, is a complex, multifactorial disease. "In the EU, there were over two million (2,288,100) incident cases of cancer in 2006 and over one million cancer deaths (1,165,500). Prostate cancer was the commonest form of cancer in men (301,500 incident cases, 24.1% of all incident cases) followed by lung cancer (194,400; 15.5%) and colorectal cancers (163,100 cases, 13.0%) (Ferlay et al., 2007)." Every year in the UK nearly 35,000 cases of prostate cancer are diagnosed, and prostate cancer causes 10,000 deaths each year (Cancer Research UK, 2008). Incident rates of prostate cancer vary worldwide. The highest rates are recorded at developed countries due to the wide use of early diagnosis testing (PSA test) and population screening programs (Figure 1.1). Many parts of the Asia and Africa show

lowest rates of prostate cancer since PSA testing is not widely practised. Therefore it is difficult to compare the prostate cancer incidence cases of different countries. Instead of incident rates if death rates were compared, it has been seen that in many developed countries the death rates for prostate cancer have been decreasing mainly due to early detection and improved treatment. The largest study of its kind, European Randomized Study of Screening for Prostate Cancer (ERSPC) showed that screening for prostate cancer by means of PSA test and DRE can reduce deaths by 20% (ERSPC, 2009) and this proves the rationale behind the mass screening.

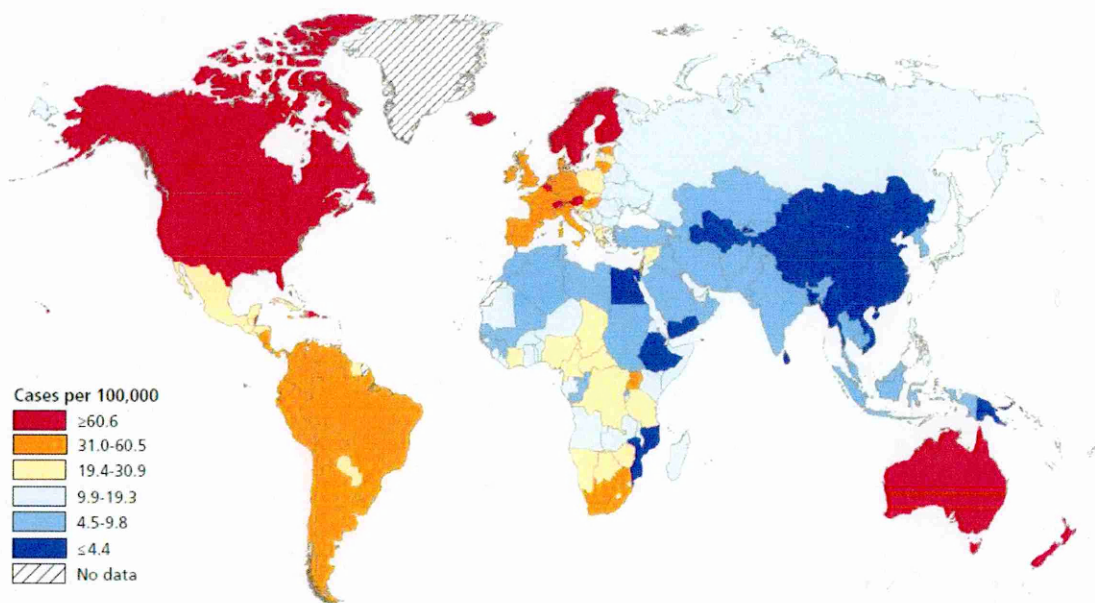


Figure 1.1. International variation in age-standardized prostate cancer incidence rates (American Cancer Society, 2007).

Earlier diagnosis and improvements of the treatment decreased the mortality rate of prostate cancer slightly in the late 1990's in UK; however, this trend did not continue for the age group 75-84 and mortality rate became stagnant in the last few years (Figure 1.2).

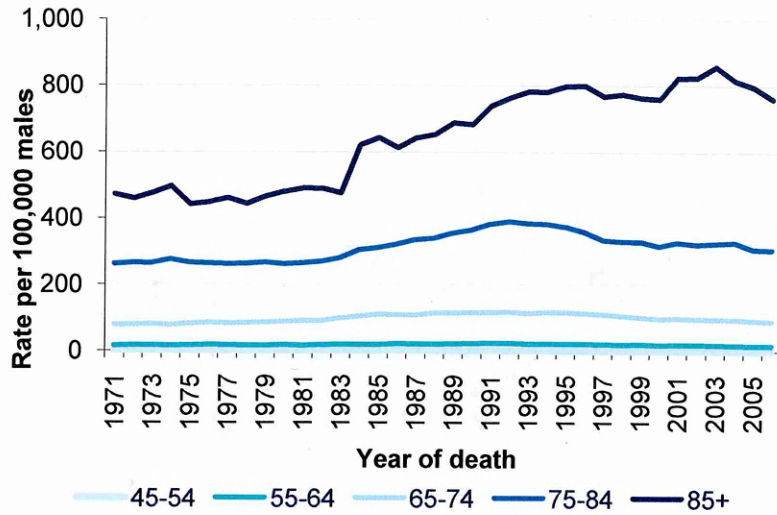


Figure 1.2. Age specific mortality rate for prostate cancer in UK from 1971 to 2006. (<http://info.cancerresearchuk.org/cancerstats/types/prostate/mortality/>)

To increase the survival rate from prostate cancer it is essential to diagnose the cancer at its earlier stages. However, there are no symptoms in the early stages of prostate cancer, and at this stage regular population screening may help to pick up patients who are in the early stages of the disease. Another problem is that, the symptoms of prostate cancer and other prostate related diseases (such as non-cancerous enlarged prostate gland or a kidney infection) are very similar and this causes confusion and makes diagnosis difficult. Early and accurate detection of prostate cancer is very important, since there is no cure if the cancer spreads out to the other organs of the body. Prostate cancer differs from person to person, while some patients require no treatment and the disease progresses very slowly, other patients suffer from a very aggressive form of cancer that require urgent clinical treatment (Bott et al., 2005). This cause confusion as early diagnosis of prostate cancer is not enough, but also type of the cancer if aggressive or not need to be

determined to decide the effective way of treating the patient. Prostate cancer can be treated using hormones, chemotherapy, radiation therapy, or surgical procedures.

1.2.2 The prostate gland

The prostate is a male sex gland located underneath the bladder (Figure 1.3). It is about the size of a walnut and fits around the tube (the urethra) which carries urine out of the bladder. The prostate produces a thick fluid that forms part of the semen. The gland on average is 3-4 cm long and 3-5 cm wide, walnut in shape with an average adult weight of 20 grams. However, due to prostatic enlargement the gland can weigh up to 150 grams (Rous, 1988).

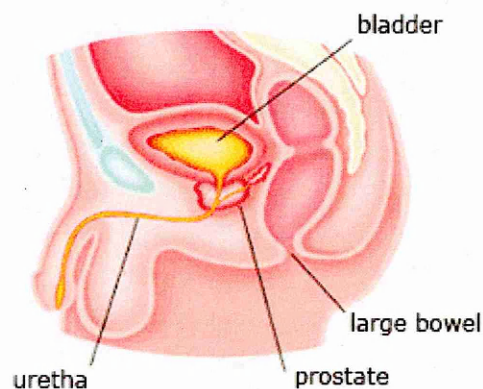


Figure 1.3. The place of prostate gland in the body (<http://www.prostate-riskindicator.com/via.html>)

Diseases of the prostate gland include:

- Benign Prostatic Hyperplasia (BPH)
- Prostatitis
- Prostatic Intraepithelial Neoplasm (PIN)
- Prostate Cancer – Carcinoma

A number of molecular changes occur during the conversion of a normal prostate epithelial cell into a malignant cell. These molecular alterations are either inherited or caused by somatic mutations (Kallioniemi and Visakorpi, 1996). It is believed that inherited factors account for 42% of the risk of prostate cancer (Lichtenstein et al., 2000). The other factors are environmental factors, geographical location, diet and race. The molecular genetics of prostate cancer is poorly understood (Elo and Visakorpi, 2001). Some of the genes that have been found to play a role in the progression of prostate cancer include GSTP1 (Gluthione S-transferase P1), PTEN (Phosphatase and Tensin homolog) and the androgen receptor (AR) gene (Visakorpi, 2003).

1.2.3 Prostate Cancer Biomarkers

The Oxford Dictionary of Biochemistry and Molecular Biology defines biomarker as “an indicator signalling an event or condition in a biological system... a biomarker may be a measurable chemical, biochemical, physiological, behavioural, or other alteration within an organism”(Cammack et al., 2006). For the diagnosis and prognosis of prostate cancer it is essential to find out the correct biomarkers. Several methods have been used for the search of potential prostate cancer biomarkers such as immunohistochemical tests, immunoassays, PCR, genomic or protein microarrays and monoclonal antibody technologies (Adam et al., 2002; Adam et al., 2001; Chuang et al., 2007; Lampe, Molkenboer-Kuenen and Oosterwijk, 2004; Miller et al., 2003; Schmidt et al., 2006). For example an antibody microarray test was conducted by Miller and colleagues to test blood samples of patients with the disease and they identified five antibodies; von Willebrand Factor, immunoglobulin M, Alpha1-antichymotrypsin, Villin and immunoglobulin G as potential prostate cancer biomarkers (Miller et al., 2003). Also a commercial DNA microarray is available

from SuperArray Bioscience Corporation. 'Oligo GEArray' Human Prostate Cancer Biomarkers Microarray' profiles the expression of 263 prostate cancer related genes. A number of review papers, covering the current and the potential biomarkers of prostate cancer have been published (Bradford et al., 2006; Shariat, Karam and Roehrborn, 2007; Stangelberger et al., 2007; Steuber, O'Brien and Lilja, 2008; Voorzanger-Rousselot and Garnero, 2007).

Due to the low sensitivity and specificity of PSA test (especially in the 4-10 ng mL⁻¹ range), it is critical to identify new biomarkers for the diagnosis of prostate cancer. The studies up to now indicate that due to the heterogeneity and complexity of the prostate cancer, multiple biomarkers are needed to be analysed together to increase the accuracy of the diagnostic assay (Laxman et al., 2008; Steuber et al., 2008). Prostate cancer biomarkers may be in the forms of antibodies, proteins, genetic sequences or enzymes/enzymatic activity and can be detected from blood or urine samples. Some of the prostate cancer biomarkers collected from the literature is listed in Table 1.1 and in the next section more detailed information is given for the selected biomarkers.

Table 1.1. Prostate cancer biomarker list.

	Prostate cancer biomarker	References
Proteins Enzyme or enzymatic activity	Prostate specific antigen (PSA)	(Brawer and Lange, 1989; Diamandis, 1998; Stephan et al., 2007)
	Human glandular kallikrein 2 (hK2)	(Becker et al., 2003; Darson et al., 1997; VŠišŠnen et al., 2006)
	Early prostate cancer antigen (EPCA)	(Dhir et al., 2004; Uetsuki and Kakehi, 2006)
	Insulin-like growth factor 1 and insulin-like growth factor binding protein 3	(Stattin et al., 2000)
	Prostate-specific membrane antigen (PSMA)	(Bander, 2006; Mhawech-Fauceglia et al., 2007; Moffett et al., 2007)
	Mini-chromosomal maintenance (MCM) proteins	(Padmanabhan et al., 2004)
	Prostate stem cell antigen (PSCA)	(Reiter et al., 1998; Zhigang and Wenlv, 2004)
	Transforming growth factor-b1	(Kattan et al., 2003; Shariat et al., 2001)
	Interleukin 6	(Lee et al., 2003)
	Urokinase-type plasminogen activator receptor forms	(Steuber et al., 2008; Steuber et al., 2007)
	Alpha-methyl coenzyme-A racemase	(Rubin et al., 2002; Sreekumar et al., 2004)
	Prostatic acid phosphatase (PAP)	(Saito et al., 2007)
	Phosphorylated AKT	(Hamdan, 2007)
	Hepsin	(Magee et al., 2001; Stephan et al., 2004)
	Telomerase activity	(Sommerfeld et al., 1996)
Genetic sequences	Prostate Cancer gene 3 (PCA3)	(Bussemakers et al., 1999; Schalken, Hessels and Verhaegh, 2003)
	2+Edel fusion	(Attard et al., 2008)
	Gluthione S-transferase P1 gene (GSTP1)	(Goessl et al., 2000; Harden et al., 2003)
Antibodies	TURP-27 (monoclonal antibody)	(Lipford and Wright Jr, 1991; Troyer, Beckett and Wright Jr, 1995; Wright Jr et al., 1991)
	Prostate cancer-specific autoantibodies	(Bradley et al., 2005; Wang et al., 2005)

1.2.3.1 Prostate Specific Antigen (PSA)

Prostate specific antigen (PSA) or in other words human glandular kallikrein 3 (hk3) is a 33-34 kDa glycoprotein and a member of the Kallikrein family (Figure 1.4).

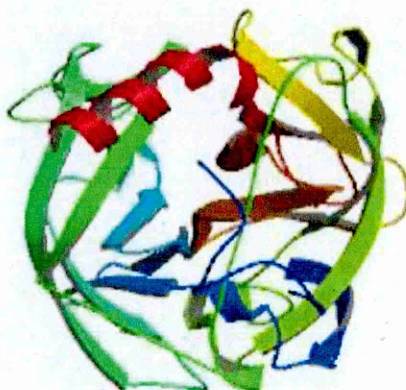


Figure 1.4. The model of the PSA protein (<http://modbase.compbio.ucsf.edu/modbase-cgi/>).

Kallikreins are serine proteases that catalyse the hydrolysis of peptide bonds. The prostatic function and production of other kallikreins and PSA are largely depend on androgen-dependent activation of the androgen receptor (Lilja, 2003). PSA is produced by the prostatic secretory epithelium cells. PSA liquefies the gel-forming proteins in semen and it is the most abundant protein in seminal plasma with a concentration of 0.2-5.0 mg mL⁻¹ (Lilja et al., 1987; Nash and Melezinek, 2000). PSA is also detectible in serum and the normal level of PSA in serum is 0.1-2.5 ng mL⁻¹.

The increase in PSA levels in serum above the normal limits is the primary indication of prostate problems which can be due to malignancy; therefore PSA is

used as a biomarker for the diagnosis and prognosis of the prostate cancer (Brawer and Lange, 1989; Diamandis, 1998). In serum PSA is found either in free form or as a complex with ACT (ACT-PSA, MW 96 kDA). ACT is a protease inhibitor and it inhibits PSA's proteolytic activity.

Although PSA is currently recognised as the clinically most used prostate cancer marker, it is not cancer specific (Stephan et al., 2007). BPH and some other non-cancerogenous diseases also cause an increase of serum PSA level. Additionally having a low PSA value does not guarantee that prostate gland is cancer free, some cancer patients have low PSA concentration in their serum. Therefore, PSA has both low specificity and low positive predictive value especially when PSA level is moderately elevated ($4-10 \text{ ng mL}^{-1}$) (Stephan et al., 2007). PSA is not only un-specific to prostate cancer but also not specific to prostate gland as well. Later studies showed that PSA has also been detected in some female tissues (including breast, ovarian and endometrial tissues) and body fluids (serum, milk, amniotic fluid) (Kamenov et al., 1998). For this reason research on PSA-based diagnostic parameters and research for new biomarkers is an ongoing study.

1.2.3.2 Human glandular kallikrein 2 (hK2)

Human glandular kallikrein 2 (hK2) was discovered in 1980s as a new member of the kallikrein family (Schedlich, Bennetts and Morris, 1987) (Figure 1.5). The studies on this new protein revealed that hK2 has 80% homology with PSA and both proteins have been found together in the biological fluids examined such as prostate and breast secretions (Darson et al., 1997; Diamandis et al., 2000).

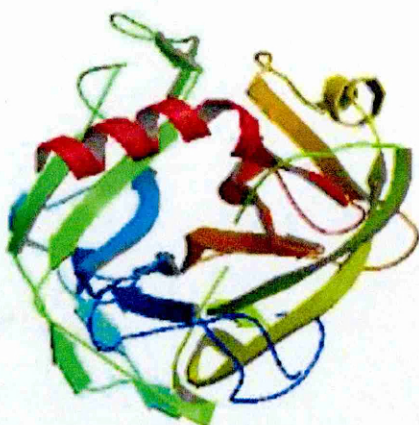


Figure 1.5. The model of human glandular kallikrein 2 protein. (<http://modbase.compbio.ucsf.edu/modbase-cgi/>)

The hK2 protein is predominantly expressed in prostate epithelial cells and present in serum. In serum and seminal plasma hK2 protein concentration is only 1-3 % of that of PSA, and this poses a challenge for the use of this protein for diagnosis (Stephan et al., 2007). For example for PSA concentrations of 2-10 ng mL⁻¹, respective hK2 concentrations are ~0.02-0.5 ng mL⁻¹ (Všišňen et al., 2004). Similar to PSA, hK2 forms complexes with some other proteins such as ACT, antithrombin III, C1-inactivator and plasminogen activator inhibitor-1 (PAI-1). In the serum, studies have suggested that 80–95% of hK2 is in the un-complexed, free form (Black et al., 1999).

Both BPH and prostate cancer results in PSA concentration increase and also PSA expression is higher in low-grade tumors compared to high-grade tumors. These conditions cause difficulty to diagnose the prostate cancer. It has been reported that hK2 concentration is higher in high-grade cancers compared to low-grade cancers and additionally the concentration of hK2 is lower in BPH (Haese, Khan and Sokoll,

2003). Therefore, hK2 has the potential to become a good diagnostic marker for the prostate cancer detection that can be used together with PSA.

Several studies have shown that hK2 concentration measurements together with tPSA or fPSA may improve the discrimination between malignant and benign prostate tissue (Becker et al., 2003; Karazanashvili and Abrahamsson, 2003). Additionally the level of hK2 concentration may help to predict prostate cancer stage and risk of biochemical recurrence after radical prostatectomy. For example Steuber and colleagues measured tPSA, fPSA and hK2 concentrations in serum samples from 461 patients who had undergone radical prostatectomy (Steuber et al., 2006). Analysis of the results showed that for the patients with a moderate tPSA level ($\text{tPSA} \leq 10 \text{ ng mL}^{-1}$), biochemical recurrence prediction was enhanced when fPSA and hK2 measurements used together. VŠisŠnen and colleagues measured the tPSA, fPSA, thK2 and fhK2 concentrations from the serum of 188 patients who had undergone radical prostatectomy (VŠisŠnen et al., 2006). It was found that hK2 could not predict the stage of the prostate cancer better than PSA; however the concentration changes of both hK2 and PSA occurred at different points in cancer progression. Therefore, VŠisŠnen suggested that hK2 might have a role in the prognosis of the prostate cancer.

Although the studies up to date have shown that hK2 has a potential as a prostate cancer biomarker, it cannot currently replace PSA test. Instead the most efficient way to combine both PSA and hK2 assays need to be investigated to improve both sensitivity and specificity of the prostate cancer diagnosis and prognosis.

1.2.3.3 Early Prostate Cancer Antigen (EPCA)

Early Prostate Cancer Antigen (EPCA) is a nuclear matrix protein and a promising prostate cancer biomarker. Two unrelated nuclear matrix proteins EPCA and EPCA-2 are considered to have association with prostate cancer. Immunohistochemical and ELISA based tests have been used and have indicated that EPCA is highly specific to malignant prostate (Dhir et al., 2004; Leman et al., 2007; Uetsuki and Kakehi, 2006). The company Onconome Inc. (USA) in collaboration with Dr. Getzenberg have developed two products called ProstaMark™ EPCA-2 Serum Assay and ProstaMark™ EPCA Tissue Assay for the diagnosis of prostate cancer (http://www.onconome.com/products_prostamark.htm).

1.2.3.4 Insulin-like growth factors and their binding proteins (IGFs)

Insulin-like growth factors (IGFs) are peptide hormones and together with their binding proteins (IGFBPs) they are part of the complex cell signalling molecules. IGFs and IGFBPs are involved in cellular proliferation, cellular transformation, growth regulation and cell death (apoptosis) (Stangelberger et al., 2007). The expression of IGFs and IGFBPs of prostatic tissue and plasma levels of IGF-1 and IGFBP-3 have been examined by researchers and some correlation was found between these markers and the prostate cancer (Peng et al., 2008; Sciarra et al., 2008; Stattin et al., 2000; Thrasher et al., 1996). However, the results are not conclusive enough and more research is needed in this area.

1.2.3.5 Prostate Cancer Gene 3 (PCA3)

In the early 1990s, Marion Bussemakers discovered a new gene that is over-expressed in malignant prostate tissue. Initially the gene was called DD3, referring to differential display technique (Bussemakers et al., 1999). Later on the nomenclature

for DD3 was changed to PCA3 (Prostate CAncer gene 3). It was found that PCA3 is a non-coding RNA that is located at chromosome 9q21-22. Further studies revealed that PCA3 is prostate tissue specific and although the biological function of PCA3 is yet unknown, still can be used as a tool to diagnose prostate cancer (De Kok et al., 2002; Schalken et al., 2003). PCA3 mRNA level can be detected in urine samples that are taken after the digital rectum examination.

1.2.3.6 2+Edel fusion gene

Recently, a new prostate cancer marker “2+Edel” was described by the teams of Professor Colin Cooper at the Institute of Cancer Research and Professor Jack Cuzick at the Wolfson Institute of Preventive Medicine (Attard et al., 2008). 2+Edel represents the fusion of TMPRSS2 to ERG genetic sequences. It was found that the patients who have 2+Edel have the aggressive form of the prostate cancer and only 25% of the patients survive after eight years. The patients who lack the alterations in the same region of their DNA have 90% survival rate in the same time period. It is believed that 2+Edel will help the clinicians to identify the patients who have the aggressive form of the cancer and need urgent treatment.

1.2.3.7 Telomerase activity

Determination of telomerase activity can be used to differentiate benign and malignant prostate and it is a potential diagnostic biomarker for prostate cancer (Ozen et al., 1998; Scatks et al., 1997; Vicentini et al., 2004). The telomeres are regions of repetitive DNA sequences at the end of chromosomes. These sequences protect chromosomes at both ends and as they are shortened with each somatic cell division they lead the cell death. In the malignant cells, telomerase adds new DNA sequences to the end of the chromosomes and thus the shortening of telomere is

prevented causing immortality of malignant cells. The researches have been shown that telomerase activity was observed in prostatic cancer cells but not in normal or BPH tissues (Kallakury et al., 1997; Lin et al., 1997; Sommerfeld et al., 1996). Botchkina and colleagues analysed the urine samples of 56 patients after DRE using quantitative PCR technique and found that all prostate cancer patients revealed high levels of telomerase activity showing 100% assay sensitivity (Botchkina et al., 2005).

1.2.4 Current diagnosis techniques

Early diagnosis of prostate cancer is very important for successful treatment of the disease. Prostate Specific Antigen (PSA) test, Prostate CAncer gene 3 (PCA3) test, rectal examination, ultrasound scan and biopsy are the current methods that are used to diagnose the malignancy of the prostate. These tests, however, are not individually conclusive. Rectal examination, ultrasound scan and biopsy are very invasive tests and have complications, however; currently there are no tests available that can avoid these methods to confirm the results. Further research is needed to come up with a test to identify the malignancy without the need of these invasive examinations. Multiplex testing of more than one biomarker may provide a better understanding of the patient's condition and may help eliminate invasive examinations. Thus, work on biomarker research and techniques enabling detection of multiple biomarkers have significant importance.

In the next part of this section, detailed information is given for the current prostate cancer detection methods.

1.2.4.1 Prostate Specific Antigen (PSA) test

PSA test is a simple blood test that measures the total amount of the PSA protein in the bloodstream. PSA test alone does not give enough information to diagnose the prostate carcinoma; if the serum PSA level is found above the recommended value (4 ng mL⁻¹), it is followed by a digital rectum examination (DRE) to make the decision of the dismissal of the patient or further biopsy analysis. Some doctors recommend yearly screening for men over the age of 50. The screening is recommended to start from age 40-45 if there is prostate cancer in the family history.

The history of PSA test first started with the characterisation of PSA in 1979 (Wang et al., 1979) and then ELISA (Enzyme-Linked ImmunoSorbent Assay) based test to detect PSA in blood or serum was developed. The use of the PSA protein as a diagnostic marker was approved by the Food and Drug Administration (FDA) in 1986 (Diamandis, 1998) and many commercial PSA test kits started to be used for the detection of prostate cancer. Three types of commercial PSA kits are available:

1. Membrane strip tests such as PSA SEMIQUANT Membrane Test (Seratec, Germany) and Pasante SelfCheck Home Prostate Health Test (Pasante Healthcare, UK) give qualitative results and mainly used for home/personal use.
2. ELISA based PSA tests are used by clinicians to get quantitative results and examples to commercial kits are Tandem-R assay (Hybritech, USA), Tosoh AIA assay (Tosoh Bioscience, Japan), AxSYM PSA assay (Abbott Laboratories, USA) and Immunolite assay (Diagnostic Products Corporation, USA).

3. Point-of-care PSA tests such as PSAwatch™ (Mediwatch Plc., UK) and FastPack™ Total/Free PSA Immunoassay (Qualigen Inc., USA) offer fast quantitative results for clinicians.

In the past, PSA concentration in serum was considered normal if it is below 4 ng mL⁻¹ (Cooner, 1990). Further studies on PSA by different groups indicated that it is possible to diagnose prostate carcinoma at a PSA level of 2.5 ng mL⁻¹ since some patients do have cancer although their PSA level is in the range of 2.5 to 4 ng mL⁻¹ (Labrie et al., 1999; Thompson et al., 2004). The use of a higher PSA cut off value of 4 ng mL⁻¹ results in the risk of missing some cancer patients; whereas using the lower threshold value of 2.5 ng mL⁻¹ may increase the number of unnecessary biopsies. Therefore, the debate about the threshold value of PSA level continues to date (Hamdan, 2007). It is stated on the USA National Cancer Institute web site that many doctors in the USA are now using the following PSA ranges with some variation

(<http://www.cancer.gov/cancertopics/factsheet/Detection/PSA>):

- 0 to 2.5 ng mL⁻¹ is low
- 2.6 to 10 ng mL⁻¹ is slightly to moderately elevated
- 10 to 19.9 ng mL⁻¹ is moderately elevated
- 20 ng mL⁻¹ or more is significantly elevated

The other weakness of the PSA test is that PSA is not specific to prostate malignancy and serum PSA concentration elevation can also be observed due to BPH, age or inflammation. To decrease the number of unnecessary biopsies, new PSA-based

diagnostic parameters have been developed by researchers such as PSA complexes level, PSA density, PSA velocity and age adjusted PSA.

In serum, PSA is found either in free form (fPSA) or bound to α -1-antichymotrypsin (ACT) as a complexed PSA (cPSA). Some of the commercial assays measure only total or free PSA assays; some others measure all three fPSA, cPSA and tPSA (total PSA). tPSA refers to the sum of all detectable fPSA and complexed PSA forms in the serum. The proportion of fPSA to ACT-PSA complex varies according to the type of the prostate cancer (Lilja, 2003). For example “significantly higher percent fPSA are found in serum/plasma from men with benign gland enlargements, whereas much more pronounced elevations of PSA–ACT levels in serum/plasma from men with prostate adenocarcinomas contribute to typical findings of significantly lower percent fPSA in these samples (Lilja, 2003)”. The studies up to date give an initial indication that measuring the level of cPSA rather than tPSA and percentage of fPSA gives an improved specificity for prostate malignancy diagnosis (Hamdan, 2007).

PSA velocity is another PSA based parameter that indicates the change in PSA concentration over time. In 2006 Carter and colleagues published a paper where they give evidence that PCA velocity determination may help to identify life-threatening form of prostate cancer (Carter et al., 2006). The study was conducted using data from 980 men, and it was concluded that the men who had a PSA velocity above 0.35 ng nl^{-1} per year had a higher relative risk of dying as the men who had lower PSA velocity values.

PSA density is the ratio of PSA concentration in blood to the sonographically determined prostate gland volume. The size of prostate gland influences the serum

PSA levels, by using the PSA density it is hoped to eliminate false positive results due to non-cancerous enlarged prostate gland. However, there is a risk of missing some cancer patients who have an enlarged prostate.

It is evident from years of studies that the incidence of prostate cancer increases with aging. Additionally; the level of PSA increases by age, mainly due to the enlarged prostate gland. Therefore, it is critical to differentiate PSA level increase due to cancer or due to aging. For this reason Oesterling and colleagues have developed an age specific reference ranges (Oesterling et al., 1993). The recommended PSA reference cut off values by Oesterling is listed below:

- The men aged 40 to 49 years, 0.0 to 2.5 ng mL⁻¹
- 50 to 59 years, 0.0 to 3.5 ng mL⁻¹
- 60 to 69 years, 0.0 to 4.5 ng mL⁻¹
- 70 to 79 years, 0.0 to 6.5 ng mL⁻¹

For over 20 years PSA has been used as a biomarker for prostate carcinoma. Due to over-diagnosis and false positive test results as being not very specific, PSA has been increasingly criticized by the researchers and doctors alike. In 2004 Stamey and colleagues published a paper with a title 'PSA era in US is over for prostate cancer' and they stated in the paper that 'in the last 5 years serum PSA has only been related to benign prostatic hyperplasia' (Stamey et al., 2004). This paper also was a call to researchers to increase the endeavours to find new serum markers that not only can diagnose the prostate cancer but also can determine the stages of cancer. Recently two papers were published by Shariat *et al.* and Schröder *et al.*; which clearly indicate that although the PSA test is not perfect, currently there is no alternative

method for prostate cancer diagnosis other than tackling the challenges around this test (Schröder et al., 2008; Shariat and Karakiewicz, 2008) .

1.2.4.2 Digital Rectal Examination (DRE)

DRE is one of the most important tests to detect prostate cancer at early stages (Van Der Crujisen-Koeter et al., 2001). In this examination a physician uses his finger to feel the prostate gland that is in front of the rectum. DRE test individually is not enough for diagnosis and always accompanied with a PSA or a PCA3 test. Most early prostate cancers has no or little symptoms, therefore for early detection of the malignancy, an annual DRE test is recommended to every man who is over 50 years of age (Van Der Crujisen-Koeter et al., 2001). If a man is at higher risk for prostate cancer (such as cancer history in the family), the annual DRE need to start earlier in life.

1.2.4.3 Prostate CAncer gene 3 (PCA3) test

Prostate CAncer gene 3 (PCA3 or differential display code 3; DD3) is a non coding gene that is over expressed in malignant prostate (Bussemakers et al., 1999). PCA3 assay was first commercialised by DiagnoCure in 2002 with the name uPM3™. In November 2003, DiagnoCure and Gen-Probe Inc. signed a licence and collaboration agreement. Later in 2006 uPM3™ was withdrawn from the market and Gen-Probe Inc. introduced its PCA3 based test (CE-marked, called PROGENSATM PCA3 Assay in Europe) (DiagnoCure). A number of laboratories in US and UK have already started to use PCA3 test routinely to diagnose prostate cancer.

PCA3 test comprises the detection of both PCA3 and PSA mRNA levels in urine samples that are taken after the DRE. PCA3 and PSA mRNA concentrations are

measured and the ratio of both results is determined as a PCA3 score ($\text{PCA3/PSA mRNA} \times 1000$). Normal prostate cells express low levels of PCA3. If only PCA3 concentration is used, depending on the amount of prostate cells used for the test, the PCA3 concentration would be high and indicate a false positive result. Thus, the concentration of PSA mRNA is used to normalise the PCA3 concentration (Torres and Marks, 2006). The clinical tests up to date indicate that a PCA3 score cut-off of 35 provides the greatest diagnostic accuracy (Figure 1.6). (<http://www.pca3.org/pro/pca3-utility>, 2010)

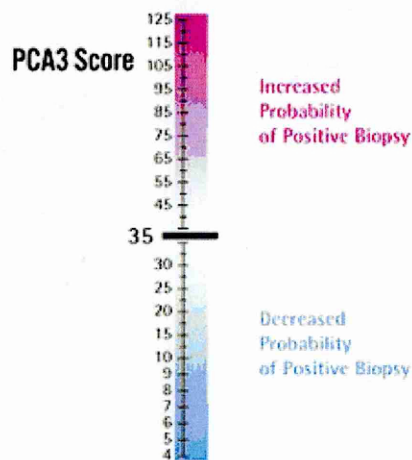


Figure 1.6. Interpretation of the PCA3 Score after PROGENSATM PCA3 Assay results obtained (<http://www.pca3.org/pro/pca3-utility>, 2010).

The clinical tests up to date indicate that as the PCA3 score increases, the likelihood of prostate cancer increases and PCA3 test performs better than PSA alone test to diagnose malignant prostate (Fradet et al., 2004; Groskopf et al., 2006; Kirby, 2007; Marks et al., 2007; Sokoll et al., 2008; Van Gils et al., 2007). Fradit and colleagues found that PCA3 test has a positive predictive value of 75% compared with 38% for total PSA and the negative predictive value was 84% compared with 80% for total PSA (Fradet et al., 2004). Graskopf *et al.* found that while prostate volume

increases, PSA concentration increase; whereas PCA3 is not affected by prostate volume (Groskopf et al., 2007; www.PCA3.org) (Figure 1.7). The larger prostate volume due to BPH or other non-cancerous conditions increase the PSA concentration of the prostate gland and thus the patients are recommended to take a biopsy test unnecessarily. As PCA3 score is not affected by prostate volume, this will decrease the number of patients sent to biopsy. However, this test is carried out after a digital rectal examination has taken place and therefore can also be called as being an invasive procedure.

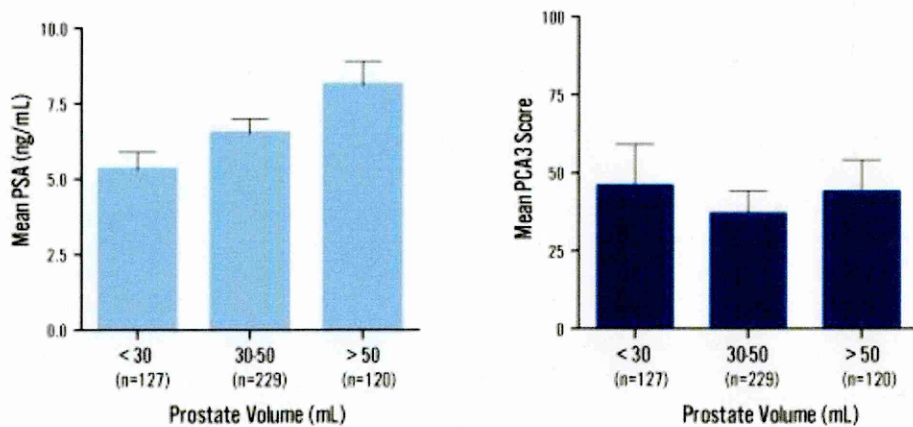


Figure 1.7. Relationship between prostate volume increase and its effects on the level of PSA and PCA3. (Groskopf et al., 2007; www.PCA3.org)

1.2.4.4 Transrectal Ultrasound Guided Biopsy

Patients with an abnormal prostate, elevated PSA (greater than 4 ng mL^{-1}) or high PCA3 score are directed to the Transrectal Ultrasound (TRUS) guided biopsy to understand the nature of the problem. TRUS guided biopsy takes 5-10 minutes and the patient is awake throughout the process. In this examination a probe with a mounted needle is inserted into the patient's rectum. The sound wave emissions produce an ultrasound image of the prostate gland; this image is used both for

calculating the volume of the prostate gland and finding the problematic sites of the gland (Applewhite and Hall, 2001). With the guidance of this image, the probe is directed to the problematic sites and samples were taken to be sent for histological analysis. Less than 1% of total prostate tissue is examined using this technique, and this may cause false negative result as some cancer may be missed. If no conclusive result is obtained, TRUS guided biopsy needs to be repeated.

The samples taken by the biopsy are analysed under a microscope by a pathologist. To interpret the biopsy specimen, Gleason scoring system is used. This grading system for prostate carcinoma is developed by Dr. Donald Gleason in 1974 as a method to distinguish the aggressive form of cancer from the less aggressive forms (Gleason, Mellinger and Ardring, 1974). Normal prostate cells are well differentiated and in general grow slowly. Whereas prostate cancer cells are disorganised and form abnormal patterns and poorly differentiated. By visually checking the level of differentiation and disorganisation it is possible to distinguish the aggressive forms of cancer from the non-aggressive forms. Each area of the cancer on a sample is given a grade of 1 through 5. The most common grade and the highest grade are added to each other to form the Gleason score that ranges from 2 to 10. Low Gleason score indicates that the tumour is not aggressive and it is less likely for the tumour to spread outside of the prostate; as the Gleason score increases the risk of more aggressive form of cancer increases (Figure 1.8) (Odowd et al., 2001).

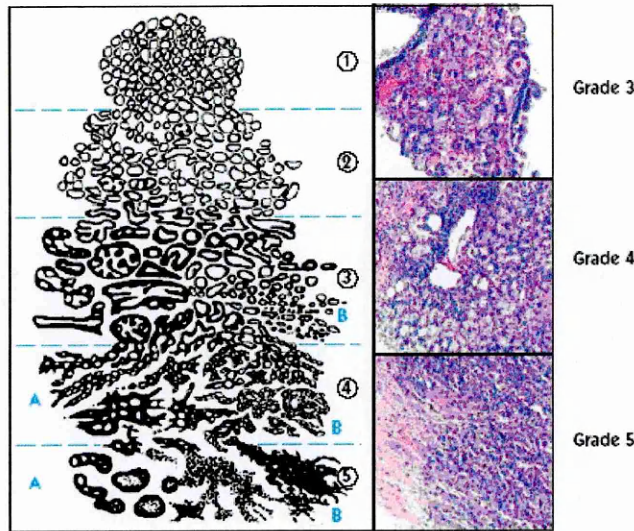


Figure 1.8. Gleason grading system diagram. Depending on the organisation and differentiation of the cells the sample is graded from 1 to 5, higher score indicating higher disorganisation and lower differentiation of the cells (Odowd et al., 2001).

1.2.4.5 Diagnosis methods under development

As described earlier, the current PSA test requires a blood sample and is not a very specific test for cancer detection. PCA3 test requires urine sample, however the sample need to be obtained after DRE examination. Another option is to detect cancer from semen sample. PSA level in semen changes (PSA concentration in semen is around 10^6 times more than in serum, (Schieferstein, 1999) from day to day depending on activities and prevent the observation of changes due to cancer. On “March 10, 2006 Proteome Systems Ltd. announced the signing of an agreement with the New York-based biotechnology company, Egenix Inc to co-develop a semen-based diagnostic kit for prostate cancer based on the known proprietary Human Carcinoma Antigen (HCA)” (http://www.psa-rising.com/wiredbird/proteomesementest_0306.html). However a recent search on the subject reveals no more information about this project suggest that either it was not

succeeded or work is in progress. Instead of HCA detection in semen, Egenix Inc develops a cPSA test kit for prostate cancer detection in serum (http://www.egenixinc.com/test_site/diagnostics.htm).

In a recent study Pal and colleagues report that citrate levels in malignant prostate cancer tissue are reliably lower and may provide information about the screening and prognosis of prostate cancer (Pal, Parker and Costello, 2009). Therefore, Pal and colleagues tried to detect lactate and citrate using luminescent ratiometric methods in microlitre samples of human serum, urine or prostate fluids. The authors are hopeful about this rapid prostate cancer test (<5 min) and more work is in progress. In another recently published study, Sreekumar and colleagues examined the potential role of sarcosine in prostate cancer progression (Sreekumar et al., 2009). They showed that the detection of sarcosine levels in urine may indicate the aggressiveness of the cancer and help to develop therapeutics for the disease.

1.3 BIOSENSORS

Biosensors are analytical devices that comprise a biological recognition element and a suitable transducer, which are usually coupled to an appropriate data processing system (Figure 1.9) (Lowe, 1985). The biological recognition element may be an enzyme, micro-organism, tissue or bioligand such as antibodies and nucleic acids (Tothill et al., 2001; Tothill and Turner, 2003; Turner, 2007). The transducer converts the physico-chemical change due to the interaction of molecules with the receptor into an output signal. Optical, electrochemical, calorimetric, magnetic, micromechanical and piezoelectric transducers can be employed in biosensors. Recognition elements can be immobilised on sensor support or sensor surface using

different methods such as; entrapment, encapsulation, adsorption, capture and covalent attachment.

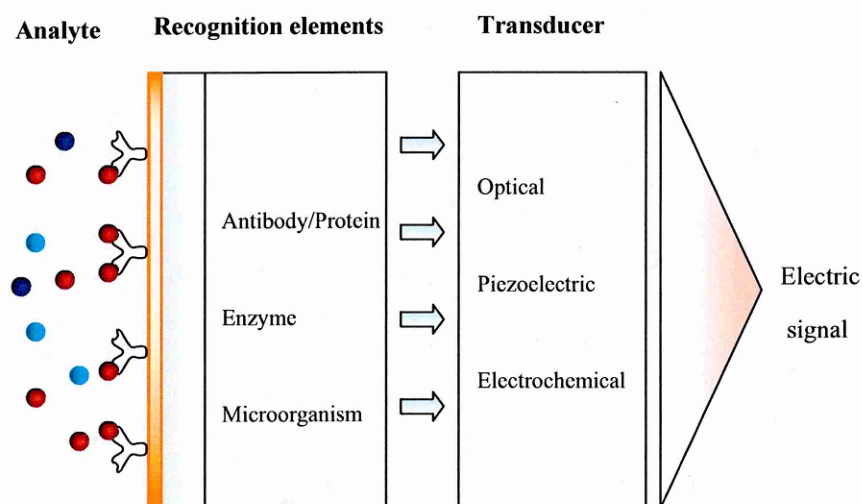


Figure 1.9. Schematic of a biosensor.

Biosensors were first described by Clark and Lyon in 1962, who described an amperometric enzyme electrode for glucose (Clark and Lyons, 1962). Shortly after this seminal paper, first glucose, then lactate and alcohol biosensors were commercialized in 1973-1975 by Yellow Springs Instrument (Newman and Turner, 2005). In 1987 a ‘pen style’ glucose sensor was launched by MediSense, which has subsequently become one of the most popular glucose biosensor (Malhotra and Chaubey, 2003). Several other glucose biosensors have been developed since and more than 40 different brands are available on the market now (Newman and Turner, 2005; Wang, 2001). While biosensors for clinical diagnostic measurements of glucose biosensor have been developed and commercialized; generic biosensors for the analysis of different molecular interactions have been developed principally only for research purposes. These types of biosensors employ sensor surface which is

ready for the immobilisation of biomolecules of interest for further biomolecular interaction analysis. The main application areas include:

- Quality assurance in agriculture, food and pharmaceutical industries
- Monitoring environmental pollutants & biological warfare agents
- Medical diagnostics
- Research and development (proteomics, drug discovery)

Biosensors can be classified according to the recognition element as bioaffinity sensors, enzyme sensors, trans-membrane sensors and whole cell biosensors; or according to the transducer as electrochemical, optical, piezoelectric and calorimetric sensors. Bioaffinity sensors provide real time measurements to detect binding of biomolecules to each other. The antibody antigen (immunosensor) (Gizeli and Lowe, 1996), receptor ligand, DNA and RNA to nucleic acid / protein binding interactions can be detected by means of bioaffinity sensors (Tothill, 2003). While preferred method of detection for catalytic sensors are usually electrochemical, the main method of detection for bioaffinity sensors are generally optical or piezoelectric (Turner, 2000).

1.3.1 Optical biosensors

Optical biosensors measure refractive index change due to binding of molecules on the sensor surface. Some of the optical methods that commercially exploited are surface plasmon resonance (SPR), diffractive optics technology, resonant waveguide grating and biolayer interferometry (Cooper, 2006). At the beginning of 1990's the commercialization of surface plasmon resonance as Biacore brand name (GE Healthcare, Uppsala, Sweden) has revolutionized the use of label free, real time biosensors in the laboratories and still dominates the biosensor market in the life sciences (Cooper, 2006). Surface plasmon resonance occurs when total internal

reflection of light happens at a metal film-liquid interface. Surface plasmon resonance causes light energy to be lost to the metal film and thus reflected light intensity decreases at a certain resonance angle. This resonance angle is dependent on local refractive index of the sensor surface (Fagerstam et al., 1992). Binding of molecules at the sensor surface changes the refractive index that can be converted to a real time electrical signal using surface plasmon phenomenon (Figure 1.10).

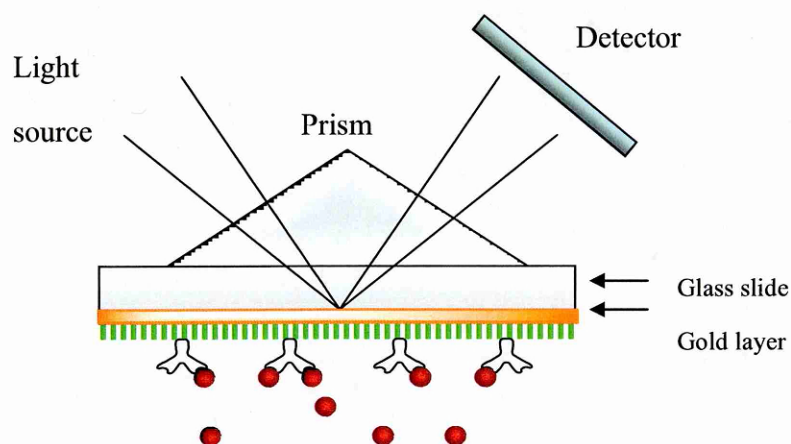


Figure 1.10. Schematic of an SPR detector and sensor chip.

1.3.2 Piezoelectric biosensors

Quartz crystal is a piezoelectric material which mechanically oscillates if an alternating voltage is applied. A quartz crystal microbalance (QCM) consists of a thin quartz disk sandwiched between a pair of electrodes. The mode of oscillation depends on the cut and geometry of the quartz crystal. If mass is applied on to the surface of the quartz resonator, the frequency of the oscillation slows down. By measuring the change of frequency, it is possible to determine the change in mass. Measurement of mass by using quartz crystal resonators was first examined by

Sauerbrey (Sauerbrey, 1959), who showed that the frequency change of the crystal resonator is a linear function of the mass per area m_s , or absolute mass Δm :

$$\Delta f_m = -\frac{f_0^2}{F_q \rho_q} m_s = -\frac{f_0^2}{F_q \rho_q} \frac{\Delta m_s}{A_{el}} \quad (\text{Eq. 1.1})$$

f_0 is the resonance frequency of the unperturbed quartz resonator, F_q the frequency constant of the crystal ($F_q = f_0 \cdot d_q$), d_q the thickness, ρ_q the quartz density, and A_{el} the electrode size of the crystal resonator. The above equation is only valid for thin, solid layers deposited on the resonator.

Initially, the QCM system was used for dry measurements, later on when suitable oscillator circuits were developed, it was possible to carry out measurements under liquid conditions (Nomura and Okuhara, 1982). This method led to the use of QCM systems as biosensors to detect molecular interactions. A new equation was derived by Kanazawa to explain the relationship between density (ρ_l) and viscosity (η_l) of the liquid and the frequency of the quartz crystal resonator:

$$\Delta f = -f_0^{3/2} \sqrt{\frac{\rho_l \eta_l}{\pi \rho_q \mu_q}} \quad (\text{Eq. 1.2})$$

where ρ_q and μ_q are the quartz density and shear modulus, respectively (Kanazawa, 1997). In a two layer system these frequency shifts simply add up to an overall shift:

$$\Delta f = \Delta f_m + \Delta f_l = -f_0^2 \left(\frac{\Delta m_s}{F_q \rho_q A_{el}} + \sqrt{\frac{\eta_l \rho_l}{f_0 \pi \mu_q \rho_q}} \right) \quad (\text{Eq. 1.3})$$

In addition to the frequency shift, there also exists a dampening of the resonator caused by the viscous liquid layer.

A quartz crystal resonator is thus not only sensitive to mass but also to viscosity and density changes on the resonator surface. Therefore the term quartz crystal microbalance is not strictly accurate for all applications. The device is also known as thickness-shear mode (TSM) resonator or a Bulk Acoustic Wave (BAW) sensor, since bulk of the crystal oscillates at a resonance frequency in a thickness shear mode of vibration.

Surface acoustic wave (SAW) sensors are also based on the piezoelectric properties of quartz crystal. In this case only a surface wave is generated by the electrodes and the frequency of the surface waves is about 100 MHz-1GHz. These frequencies are much higher than TSM resonators, and this is the reason for the higher sensitivity of SAW sensors. However, higher sensitivity also means higher response to viscosity changes and this problem causes difficulties when SAW sensors are used in liquids (Janshoff, Galla and Steinem, 2000).

The number of publications that involve the use of QCM has increased rapidly since the mid 1980s and in particular since the 1990s. There has been a steady growth in the number of published papers describing detection of clinically relevant analytes and, in the last five years, rapid growth in the number of publications on the analysis of DNA from pathogenic organisms (Caruso et al., 1998; Hook et al., 2001), viruses, (Susmel, O'Sullivan and Guilbault, 2000; Zhou et al., 2002) bacteria (Pavey et al., 2001) and mammalian cells (Richert et al., 2002; Wegener et al., 2000).

Many of the commercial QCM devices do not have thermal control units and rely on experiments using static cells. As QCM measurement is very sensitive to thermal

changes, these cause drifting baseline and difficulty to measure frequency changes due to the assay. Additionally, if there is no stirring of the sample in a static cell, the molecular interaction takes longer time due to the mass transport limitations. Instruments using flow based systems may have problems during sample injection as everything need to be performed manually and minute changes of the flow rate during this process causes baseline disturbances or drift. Recently Sierra Sensors GmbH (Hamburg, Germany) launched the first prototype of QCMA-1 biosensor, a single unit bench top QCM instrument, to overcome the problems stated above. QCMA-1 instrument has fully automated sample processing unit enabling unattended run of samples, proprietary continues microfluidic sample delivery technology called Hydrodynamic Isolation™ and an environmentally controlled detection chamber that provides maximum thermal control. A collaboration study with Sierra Sensors GmbH has been started to test QCMA-1 biosensor and its applicability for PSA detection.

1.3.3 Electrochemical biosensors

The first biosensor described was an electrochemical biosensor for the detection of glucose by Clark and Lyon in 1962 (Clark and Lyons, 1962). The first commercial biosensors (glucose, lactate and alcohol biosensors) were also reported using electrochemical techniques (Newman and Turner, 2005). High sensitivity, selectivity, rapid analysis, ability to operate in turbid solutions and the possibility of miniaturization enabled electrochemical biosensors to become the most commonly used biosensors over other technologies (Shah and Wilkins, 2003). Electrochemical biosensors may be divided into three main categories depending upon the electrochemical property measured by a detector system; conductometric, potentiometric, amperometric (Table 1.2).

Table 1.2. Types of electrochemical transducer for classified types of measurement, with corresponding analytes to be measured (Bergveld and Thea venot, 1993).

Measurement type	Transducer	Transducer analyte
Potentiometric	Ion-selective electrode (ISE);	K^+ , C^- , Ca^{2+} , F^- ;
	glass electrode;	H^+ , Na^+ ...;
	gas electrode;	CO_2 , NH_3 ;..
	metal electrode.	redox species
Amperometric	Metal or carbon electrode	O_2 , sugars, alcohols....;
	chemically modified electrodes	sugars, alcohols, phenols, oligonucleotides...
Conductometric, impedimetric	Interdigitated electrodes metal	Urea, charged species,
	electrode	oligonucleotides ...
Ion charge or field effect	Ion-sensitive field-effect	H^+ , K^+ ...
	transistor (ISFET);	
	enzyme FET (ENFET)	

The majority of commercial biosensors available on the market are amperometric enzyme biosensors (Mulchandani and Rogers, 1998). Amperometric detection is based on measuring the current generated by the oxidation or reduction of an electroactive species at a working electrode while the potential is kept constant. Two or three electrodes may be used for amperometric sensors; working electrode (usually platinum or gold), reference electrode (usually Ag/AgCl) and counter (or auxiliary) electrode (usually carbon).

Amperometric biosensors are based on oxido-reductase enzymes such as; glucose oxidase (Shan et al., 2008; Wu et al., 2007), lactate oxidase (Pfeiffer et al., 1997), glutamate oxidase (Zhang, Mullens and Gorski, 2006), galactose oxidase (Hasebe

and Uchiyama, 2000) or xanthine oxidase (Agui et al., 2006). Oxido-reductases use molecular O_2 as an electron acceptor and produce H_2O_2 in the reaction with their substrates (Figure 1.11).

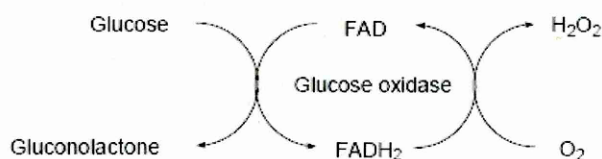


Figure 1.11. Oxidation of glucose by glucose oxidase.

The amperometric biosensors based on oxido-reductases are very sensitive to dissolved oxygen concentration of the solution that may fluctuate due to pH, temperature or other physical environment of the biosensor (Mulchandani and Rogers, 1998). As a solution to this problem, the electron acceptor O_2 is replaced by low molecular weight mediators, such as ferrocene (ϵ 5-bis-cyclopentadienyl iron), tetrathiafulvalene (TTF), tetracyanoquinodimethane (TCNQ), N-methylphenazin-5-ium cation (NMP^+) and methylene blue. Still the oxygen needs to be eliminated from the buffer and samples because oxygen is a better electron acceptor than these mediators. Eliminating the oxygen dependence of the amperometric biosensors resulted in control of the enzymatic reaction and the sensor performance (Vreeke, 1997). And hence the use of freely diffusing mediators created second generation amperometric biosensors. By the immobilisation of both the enzyme and the mediator to the electrode, a self-contained third generation amperometric biosensors are developed.

1.3.3.1 Capacitive biosensors

Detection of biomolecules with capacitive biosensors does combine the advantages of electrochemical detection with label-free bioaffinity sensing and has been used for the detection of many different biomolecules (Hedstrom, Galaev and Mattiasson, 2005; Numnuam et al., 2009; Quershi et al., 2009; Siqueira et al., 2009). The measuring principle of capacitive biosensors are based on the measurement of changes in dielectric properties and charge distribution due to the recognition element and analyte complex formed on the surface of an electrode. The capacitance (C) can be described by equation 1.4; where ϵ is the dielectric constant of the medium between the plates, ϵ_0 is the dielectric constant of free space (8.85419 pF/m), A the area of the plates and d the distance between the plates.

$$C = \epsilon \times \epsilon_0 \times A/d \quad \text{Equation 1.4}$$

In the literature several instruments were mentioned for capacitive/impedance measurements for biomolecule detection such as LCR meter (Berney et al., 1997; Klein et al., 1995; Pak, Penrose and Hesketh, 2001), potentiostat (Numnuam et al., 2009) or impedance analysers (K'Owino and Sadik, 2005). While faradaic capacitive measurements requires the addition of redox-active species, no additional reagent is needed for non-faradaic measurements (Daniels and Pourmand, 2007). As a label-free detection method, capacitive biosensors have certain advantages with respect to labelled biosensors such as the ability of direct, real-time measurement and no need of expensive labels or labelling processes. However, care need to be taken as any non-specific binding can cause a capacitive measurement signal and this need to be

distinguished from specific binding. This can be particularly problematic if the analyte need to be detected in a complex matrix such as blood serum.

Assay type can also influence the results obtained from capacitive biosensors. Generally the assays can be divided according to the measurement medium as in wet and dry conditions; or spot measurements and real-time continuous measurements. While continuous assays require the measurements to be performed in biological solutions, spot measurements can be performed in wet or dry conditions before and after the analyte binding to the recognition elements of the sensor surface. In this case reproducibility of measurements has significant importance to distinguish the results from specific analyte recognition from the standard deviation of the repeated measurements before and after removing and placing the sensor to the instrument and the connectors. Additionally the spotting of the biological materials for immobilisation and subsequent drying protocol may cause denature of some of the immobilised antibodies and may influence the sensitivity of the antigen detection.

1.4 BIOSENSOR SURFACE CHEMISTRY

The immobilisation of biological recognition elements to the biosensor surface has significant importance for the biosensor to function. While some of the optical biosensors consist of silicon oxide as sensor surface, most of the biosensor surfaces are coated with metals (such as gold, silver and platinum) for transduction of the signal. Biological recognition elements such as proteins are adsorbed spontaneously on metal or silicon oxide substrates and this may denature of the molecule and may result in either activity reduction or loss. Self assembled monolayers (SAMs) provide an effective way to protect the biological recognition element from the sensor surface, provide ligand immobilisation that enables the repeated binding and

regeneration cycles to be performed without losing the immobilised ligand and additionally help to minimise non-specific adsorption (Homola, 2006). In addition to SAMs, polymeric structures such as carboxymethyl dextran, poly-pyrrole, polystyrene, polyvinyl alcohol and polyaniline can be used to modify biosensor surfaces (Adhikari and Majumdar, 2004).

1.4.1 Self assembling of monolayers

Self assembling is defined as the spontaneous and reproducible organization of the molecules to a defined nano-scaled structure (Vericat et al., 2006). One of the most sophisticated examples of self assembly can be given as the formation of a living cell that itself contains self assembled lipid bilayer, proteins and DNA. SAMs are one of the simplest examples of self assembly and can be formed by means of silanes on oxidised and hydroxylated surfaces and by means of thiols on metal surfaces (mainly on gold). Self assembly of alkanethiols on gold substrates were first reported by Nuzzo and Allara in 1983 (Nuzzo and Allara, 1983) and since then it became one of the most used method to modify biosensor surfaces.

Each thiol molecule can be divided in three parts: the head (linking group, consist of S atom), the backbone or spacer (hydrocarbon main chain), and the terminal specific (active) group (Vericat et al., 2006) (Figure 1.12). Sulphur atom is covalently bound to metal surface and the van der Waals forces between the hydrocarbon chains stabilize the structure and create the monolayer. Terminal group of the molecule determines the surface characteristics of the formed SAM. For example $-\text{CH}_3$ and $-\text{CF}_3$ terminal groups create hydrophobic and $-\text{COOH}$, $-\text{NH}_2$ or $-\text{OH}$ groups yield hydrophilic surfaces.

A

B

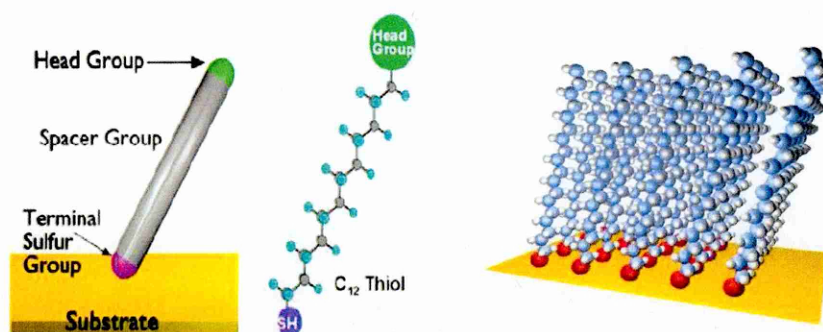


Figure 1.12. A) Parts of alkanethiol molecule. B) Self assembled monolayer of alkanethiols (Asemlon, 2005a).

SAMs can be formed on surfaces either by simply immersing the metal substrate to the dilute solution of thiol /silane or can be formed by vapour deposition (Schreiber, 2000). The most common and easiest method for coating metal surfaces with alkane thiols is the first method. As soon as the metal substrate is immersed in dilute thiol solution, a disorganised monolayer spontaneously forms and then slowly molecules are organised by means of van der Waals forces (Bain et al., 1989). Au substrates commonly used are evaporated thin films on glass or quartz substrate which forms (111) crystalline structure. The alkanethiols form a hexagonal close packed crystal lattice that has ca. $30\frac{1}{4}^\circ$ tilt and the spacing between the alkane chains is $4.97 \text{ \AA}$ (Figure 1.13).

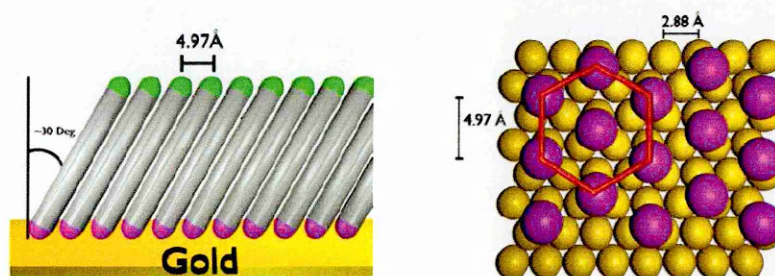


Figure 1.13. “A tightly packed alkanethiol monolayer is shown. In order to maximize the van der Waals forces between the alkane chains, the individual chains are tilted $\sim 30^\circ$ from the surface normal.” (Asemlon, 2005a)

The quality of the assembled monolayer depends on the cleanliness of the metal substrate, the purity of the alkanethiol solution and the length and composition of the alkanethiol. The head group of the alkanethiol is critical to obtain a monolayer with required functionality. Alkanethiols with different head groups are commercially available to immobilise biological recognition elements on biosensor surfaces such as $-\text{COOH}$, $-\text{NH}_2$, $-\text{OH}$, $-\text{biotin}$, $-\text{N-hydroxysuccinimide (NHS)}$.

The compactness of the monolayer depends on both the size and the structures of the spacer and the head group of the alkanethiol molecule. In order to have compact and ordered monolayer the number of carbon atoms in the spacer structure need to be 10 or more (Bain et al., 1989). To immobilise biomolecules to the sensor surface carboxylic acid head group provides not only immobilisation strategies using different reactions (Figure 1.14) but also negatively charged carboxylic acid surface causes electrostatic attraction of biomolecules and increases the yield of immobilisation.

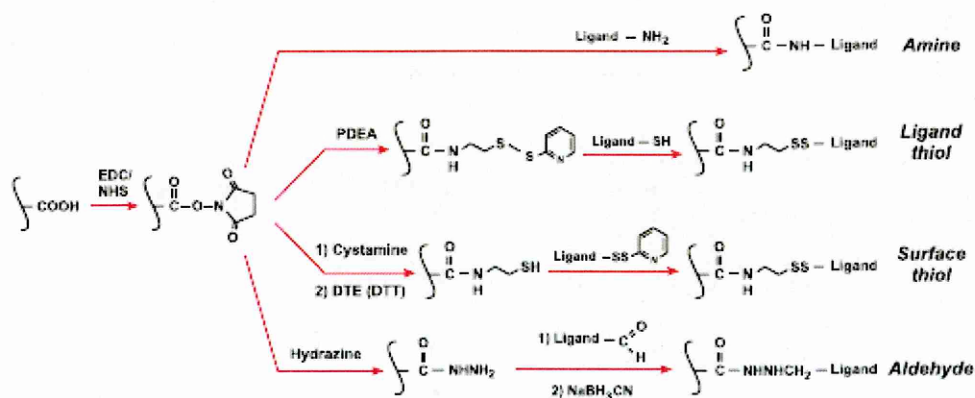


Figure 1.14. Different reaction sequences that can be applied to immobilise biomolecules to the carboxylic acid head group containing SAM (Homola, 2006).

1.4.2 Use of dendrimers to enhance biosensor surface capacity

To be able to increase the capacity of the sensing layer as an alternative to SAM surface dendrimers were considered. Poly(amidoamine) (PAMAM) dendrimers are large, branching molecules formed by repeated addition of functional groups to an ethylenediamine core (Figure 1.15). PAMAM dendrimers range in diameter from about 2 nm (generation 1) up to about 13 nm (generation 10). The molecular weight and number of peripheral groups of dendrimers increase exponentially with each generation, while the diameter increases more or less linearly. A generation 1 or generation 2 PAMAM dendrimer has an open, flat structure but generation 4 and larger dendrimers have spheroidal structure (Esfand and Tomalia, 2001). PAMAM dendrimers are reported to be robust, covalently fixed, three-dimensional structures, which provide a high density of terminal amino, carboxyl or hydroxyl groups at the outer sphere (Esfand and Tomalia, 2001). Dendrimers possess a large number of end groups per molecule and thereby increase surface functionality (Rahman et al., 2000). The properties of dendrimers make them ideal molecular binding blocks for wide range of applications involving self-assembled monolayers, which could be used for chemical sensing purposes (Singh et al., 2009; Svobodova et al., 2006; Yamaji and Takaguchi, 2009; Zhu et al., 2009). For example, a 4th generation PAMAM dendrimer having partial ferrocenyl-tethered surface groups was used as sensing layer for an electrochemical sensor by Yoon and co-workers (Yoon, Hong and Kim, 2000). Dendrimer activated solid support has been used for nucleic acid and protein microarrays by Benters and co-workers (Benters, Niemeyer and Wohrle, 2001).

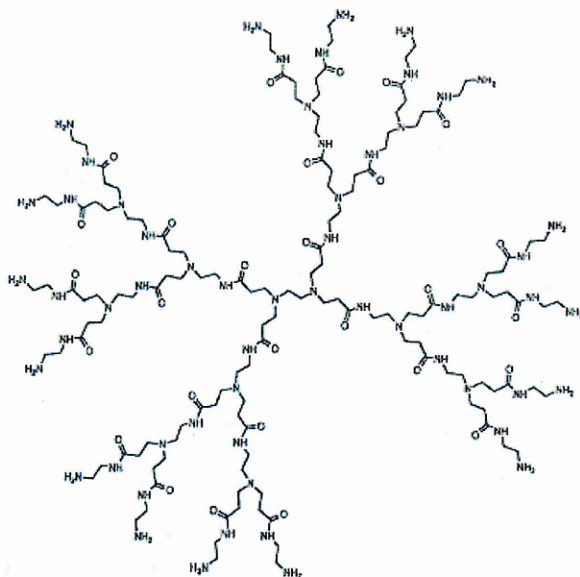


Figure 1.15. Second generation poly(amidoamine) dendrimer.

1.4.3 Free radical polymerisation for the fabrication of new polymer surfaces for sensing

To graft polymer layers on solid matrices, free radical chain polymerisation is a widely used method. In the early 1980's, Otsu et al. reported the use of new type of initiators classified as dithiocarbamate derivatives to use for free radical chain polymerization (Otsu and Matsumoto, 1998; Otsu et al., 1989). This new type of initiators was described as photoiniferters, which was an abbreviation for initiator, transfer agent and terminator. Because bimolecular termination and other transfer reactions are negligible, iniferter based polymerisation is also a living polymerisation. Upon UV irradiation, the photoiniferter undergoes a homolytic bond cleavage yielding a carbon radical and a dithiocarbamyl radical (Figure 1.16). The carbon-centered radical can react with vinyl monomers while the dithiocarbamyl radical is relatively stable and react weakly or not at all with vinyl monomers. The dithiocarbamyl radical instead reversibly terminates the growing chain by reacting

with the propagating free radicals. This reversible “capping” allows dithiocarbamate derivatives to be used as controlled free-radical initiators.

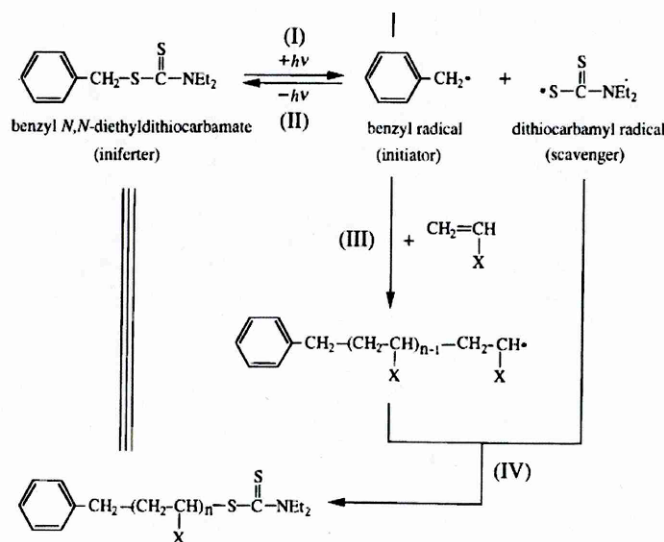


Figure 1.16. The schematics of photoiniferter based polymerisation (Nakayama and Matsuda, 1996).

Some of the advantages of polymer fabricated using initiator/iniferter based living polymerisation with respect to the other polymers used for biomolecule immobilisation (carbohydrate based hydrogels such as carboxymethylated dextran, alginate, chitosan and other conventional polymers such as polystyrene) are:

- Linear increase of molecular weight with time enables to control the thickness of the polymer growing on a substrate.
- Possibility of fabricating multiple polymer layers having different properties using different monomers.
- Compatibility with a wide variety of monomers, e.g., acrylates, styrenes, acrylonitrile, and derivatives.

- Depending on the side group of the monomer a variety of immobilisation methods can be utilised for biomolecules (for example a monomer comprising biotin side group enables capture of avidin, a monomer comprising amino side group enables immobilisation of biomolecules using any suitable homo/heterofunctional linker).
- Ease of application.
- Long shelf life.
- Enables the fabrication of rigid polymers that does not swell in buffer.
- The size of the pores can be controlled by changing the monomer/cross linker ratio.

The concentration and the ratio of monomer(s) and crosslinker(s) are crucial to obtain porous, 3 dimensional crosslinked polymer on the sensor surface. By changing the monomer(s)/crosslinker(s) ratio and concentration, porogen(s) type and amount in the polymerisation mixture, intensity and time of irradiation it is possible to obtain a polymeric structure with specified properties (such as porosity, rigidity and thickness) can be produced for different applications of sensing. By changing the functional group of the monomer, polymer surfaces with different properties may be fabricated that enables different immobilisation strategies for biomolecules.

1.4.4 Development of carboxymethyl dextran surface

Two common methods are reported in the literature to achieve CMD coated sensor surface:

1. **Epoxy-linked CMD:** Fabricating a hydroxyl end group for SAM modification on the sensor surface and attachment of dextran using epichlorohydrine. Later modification of the hydroxyl groups to carboxyl

groups by bromoacetic acid. This method was first described in 1990 by Lofas and Johnsson (Lofas and Johnsson, 1990b), it takes around 3 days to produce the sensor chips.

2. **Amide-linked CMD:** Fabricating an amino end group SAM on the sensor surface and attachment of CMD with conventional EDC/NHS chemistry. If the CMD is obtained readymade, this method is simple, contains less steps, less chemicals and short. This method can be applied in two different ways:
 - a. Incubation of CMD with amino-modified sensor surface to allow electrostatic attraction. After washing the excess material, covalent attachment of CMD using EDC/NHS chemistry.
 - b. Preparing a fresh mixture of CMD with EDC/NHS (to obtain NHS-dextran) and placing on an amino-thiol modified sensor surface for covalent attachment (Figure 1.17).

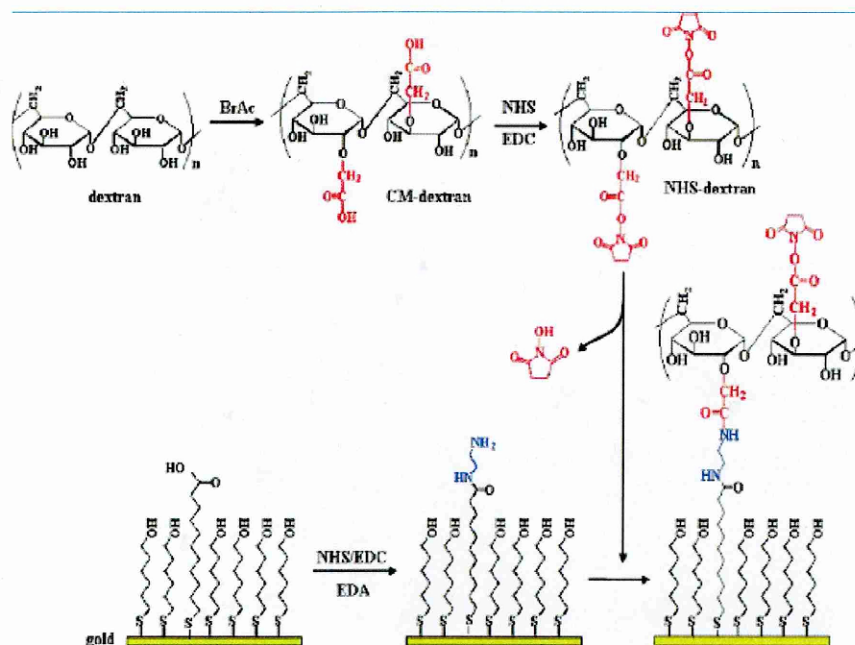


Figure 1.17. Schematic diagram for CMD surface fabrication using amide-linked NHS-dextran. (Jung et al., 2007)

Both methods described above can be used to prepare the CMD sensor chips. If the same molecular weight dextran was used, the performance of the fabricated sensor chips by both methods will depend on the polymer attachment degree to the sensor surface and the degree of carboxyl groups per glucose units of the created CMD. Jung and colleagues (2007), prepared CMD arrays on gold surfaces using both methods and compared the results. They commented that the immunoassay array results obtained using amide-linked CMD surfaces were superior to epoxy-linked CMD surfaces when line-scanning mode of the spectral SPR biosensor was used as the detection method. Due to its practicality, use of less chemicals and shorter fabrication time, amide-linked CMD fabrication on sensor chips is favourable.

1.5 PROSTATE SPECIFIC ANTIGEN DETECTION USING BIOSENSORS

PSA test is currently recognised as the clinically most used prostate cancer detection method. The current PSA tests rely on ELISA type assays and usually performed at centralised laboratories using expensive automated analyzers and time consuming procedures (Healy et al., 2007). This process requires sample transportation, increased waiting time and high medical costs. Therefore, there is a need to develop a detection system that is cheap, quick to process, uses low sample and reagent volume and easily operated. Additionally due to the weaknesses of PSA testing and complexity of prostate cancer, there is an increasing need to test multiple biomarkers simultaneously for cancer diagnosis/prognosis. Recent development and advances in biosensor technology have enabled the miniaturisation of the devices and multiplex testing of the analytes. Therefore biosensor technology has the potential for the manufacture of point of care cancer testing devices. Already two point-of-care PSA

tests entered to market as PSAwatch™ (detection range 0.5-25 ng mL⁻¹) by Mediwatch Plc. (UK) and FastPack™ Total/Free PSA Immunoassay by Qualigen Inc. (detection range 0.04 – 50 ng mL⁻¹)(USA). While PSAwatch™ is based on a lateral flow device; FastPack™ is based on chemiluminescence measurement. Both devices are not designed for simultaneous multi-analyte testing.

Several biosensor types have been applied for the detection of cancer biomarkers (Tothill, 2009). Biosensors for PSA analysis include electrochemical biosensors (Fernandez-Sanchez et al., 2004; Li et al., 2005; Liu et al., 2007; Liu, 2008; Okuno et al., 2007; Panini et al., 2008; Sarkar et al., 2002; Zhang, Du and Li, 2007b), optical biosensors (Besselink et al., 2004; Cao et al., 2006; Choi et al., 2008; Huang et al., 2005; Schweitzer et al., 2000; Yu et al., 2004), fluorescence / chemiluminescence biosensors (O'Neill et al., 1995; Soukka et al., 2003; Ye et al., 2004), microcantilever biosensors (Hwang et al., 2004; Lee et al., 2005; Wee et al., 2005) and QCM biosensors (Zhang et al., 2007a) have also been developed. In the next sections more detail is given about the different biosensing methods that applied for PSA detection.

1.5.1 PSA detection using electrochemical biosensors

Electrochemical measurements for PSA detection either can be performed label free by means of impedance and capacitance methods, or enzyme labels can be used for amperometric and potentiometric detection methods. Examples of PSA testing using electrochemical methods are summarised in Table 1.3. The detection limit of the electrochemical PSA assays varies between 0.001 ng mL⁻¹ to 5 ng mL⁻¹ and in most cases assays have been performed either in buffer or 5 to 20 times diluted human serum. To amplify the detection signal quantum dots, nanoparticles or carbon nanotubes have been employed in the assays. Electrochemical sensing has the

advantages of high sensitivity, possibility of miniaturisation and enables multiplex testing.

Table 1.3. Examples of PSA assays performed using electrochemical detection methods.

Enzyme and method used	Sensor type	Surface chemistry	Assay	Assay time	Amplification method	LOD without amplification	LOD after amplification	Serum concentration	References
GOX-amperometric	SPE	PEI, nafion, and polypyrrole	tPSA		-	0.25 ng mL ⁻¹	-	-	(Sarkar et al., 2002)
HRP-amperometric	Au-wire electrodes	Phenylboronic acid +MUDA mix	PSA	60 min	-	2 ng mL ⁻¹	-	-	(Liu et al., 2008)
HRP-amperometric	Glassy carbon electrode	Carbon nanotubes (CNTs)		30 min	CNTs	-	0.08 ng mL ⁻¹	5 times diluted serum	(Panini et al., 2008)
HRP-voltammetric	Mercury electrode	Solution is removed from the well plate and measured in an electrochemical cell	PSA	110 min	-	0.1 ng mL ⁻¹	-	Whole serum	(Zhang et al., 2007b)
Stripping Voltammetric Measurement	Immune-chromatographic strip with the electrochemical detector - SPE	-	PSA	10 min +	Nanoparticle	-	0.02 ng mL ⁻¹	20 times diluted serum	(Lin et al., 2008)
Differential pulse voltammetry	Pt microelectrode	CNTs (carbon nanotubes)	tPSA	60 min	CNTs	-	0.25 ng mL ⁻¹	-	(Okuno et al., 2007)
Conductance	-	nanowires and CNTs	PSA	-	CNTs	-	5 ng mL ⁻¹	-	(Li et al., 2005)
Impedance	SPE-microarray	polyaniline + avidin-biotin	PSA	-	-	0.001 ng mL ⁻¹	-	-	(Barton, Davis and Higson, 2008)

Enzyme and method used	Sensor type	Surface chemistry	Assay	Assay time	Amplification method	LOD without amplification	LOD after amplification	Serum concentration	References
Urease-capacitance	SPE-immunostrip	polymer	tPSA and pPSA	35 min	-	4 ng mL ⁻¹	-	-	(Fernandez-Sanchez et al., 2004)
Square-wave voltammetric (SWV)	Immuno-chromatographic strip with the electrochemical detector - SPE	-	PSA	30 min	Quantum dots	-	0.03 ng mL ⁻¹	20 times diluted serum	(Liu et al., 2007)
Cyclic voltammetry (Potential Shift Difference)	4 mm Au electrode	Au colloid-alumina sol gel	PSA	15 min	Au colloids	-	3.4 ng mL ⁻¹	5 times diluted serum	(Liu, 2008)

1.5.2 PSA detection using optical biosensors

Optical biosensors may use two different signal transduction methods such as label-based technologies (such as fluorescence or chemiluminescence biosensors) and label free technologies (such as SPR, resonant mirror (RM) and interferometric biosensors). For example Soukka *et al.* performed an immunoassay for the detection of PSA using europium (III) nanoparticle label technology and obtained 0.21 ng mL^{-1} detection limit (Soukka *et al.*, 2003). In another study Ye and colleagues used zirconia-based fluorescent terbium nanoparticles for PSA immunoassay and obtained 0.4 ng mL^{-1} detection limit with dynamic range up to ca. 10 ng mL^{-1} (Ye *et al.*, 2004). Besselink and colleagues used IBIS instrument that uses the SPR technology and after signal amplification with gold colloids obtained detection limit of 0.15 ng mL^{-1} PSA. In another study, Choi *et al.* performed PSA detection assay using an SPR spectroscopy (MultiskopTM, Optrel Gbr, Germany). For the assay with 12 hours reaction time in static conditions (in buffer) they obtained detection limit of 300 fM (0.01 ng mL^{-1}).

1.5.3 PSA detection using QCM biosensors

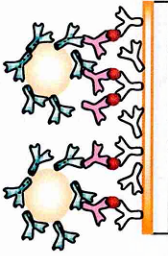
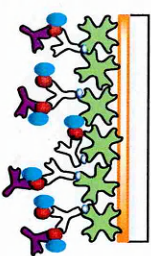
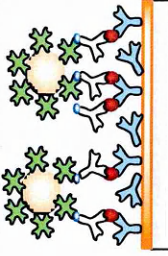
The number of publications related to the detection of PSA using QCM biosensors is very limited and this indicates that application of QCM biosensors to PSA detection is a newly developing area. An example to a study for PSA assay using QCM biosensor has been the study performed by Zhang *et al.* that involved the use of an insert-plug model of piezoelectric immunosensor. After 40 minutes of reaction at 37°C in a static cell with a 10 MHz quartz crystal, Zhang *et al.* achieved linear detection of $1.5 - 40 \text{ ng mL}^{-1}$ PSA in 100% serum (Zhang *et al.*, 2007a). In another study, Ding *et al.* used yeast cell modified QCM sensors to immobilise anti-PSA

antibodies (Ding et al., 2008). The binding of PSA to the anti-PSA antibody immobilised sensor was recorded after the binding reached equilibrium using a 9 MHz quartz crystal and magnetic stirrer agitated static cell. With this method Ding *et al.* achieved detection of PSA in the range of 5 to 604 ng mL⁻¹ in 100% serum.

1.5.4 Summary

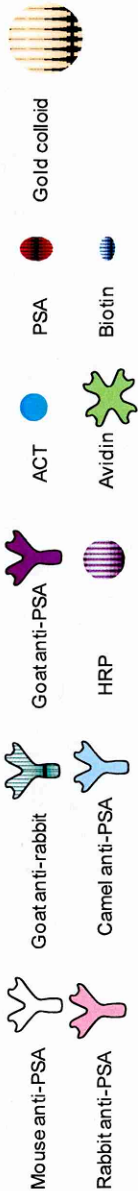
A list of the biosensor formats applied to PSA detection is given in Table 1.4. The detection limit of PSA using these systems varies between 0.2 pg mL⁻¹ to 10 ng mL⁻¹. The detection signal has been amplified by means of a sandwich assay, gold nanoparticles, carbon nanotubes or PCR for the majority of the biosensor systems. In most cases PSA assay was performed only in buffered pure solutions rather than serum, indicating the early stage of development of the technology. There is a significant difference on the limit of detection when buffer or clinical sample (serum) is used as the assay media, as it could be seen from the work of Cao *et al.* (Table 1.4). The increase of detection limit, when clinical samples are used, is mainly due to the lower signal to noise ratio resulting from the high non-specific binding of sera proteins/antibodies to the sensor surface. Therefore it is critical to create a biosensor surface that has low non-specific binding properties. Another important point that needs at most care is the selection of control sensor surfaces to prevent false negative or false positive results from the PSA assay.

Table 1.4. Examples of PSA assay in the literature.

	Biosensor type	Analyte	Assay format and/or nanostructure used	Detection limit for direct assay in buffer	Detection limit in buffer	Detection limit in serum	References	Schematics of the assay
Optical biosensors	SPR (IBIS II apparatus)	PSA	sandwich assay format, gold colloid (10nm) amplification, planar surface	60 ng mL ⁻¹	0.15 ng mL ⁻¹	-	(Besselink et al., 2004)	
	SPR (BIAcore 2000)	PSA- ACT	sandwich assay format, planar surface	20.7 ng mL ⁻¹ in buffer 47.5 ng mL ⁻¹ in 10% serum	10.2 ng mL ⁻¹	18.1 ng mL ⁻¹ in 10% serum	(Cao et al., 2006)	
	SPR (BIAcore 2000)	PSA	sandwich assay format, gold colloid (20nm) amplification, planar surface	10 ng mL ⁻¹	1ng mL ⁻¹	-	(Huang et al., 2005)	
	Surface Plasmon Fluorescence Immunoassay	PSA	sandwich assay, CMD sensor surface	-	-	~ 2pg mL ⁻¹	(Yu et al., 2004)	

	Biosensor type	Analyte	Assay format and/or nanostructure used	Detection limit for direct assay in buffer	Detection limit in buffer	Detection limit in serum	References	Schematics of the assay
Piezoelectric sensors	Piezoelectric nanomechanical microcantilever	PSA	direct assay, planar surface (Calixcrown SAM)	0.01 ng mL ⁻¹	-	-	(Lee et al., 2005)	
	QCM multi-array	free PSA	direct assay, planar surface	1.5ng mL ⁻¹	-	-	(Zhang et al., 2007a)	
Electrochemical biosensors	Amperometric sensor using SPE	total PSA	sandwich assay, amperometric immunosensor	-	0.25 ng mL ⁻¹	-	(Sarkar et al., 2002)	
	Potentiometric electrochemical immunosensor	PSA	direct assay on gold colloids/alumina derived sol-gel film	3.4 ng mL ⁻¹	-	-	(Liu, 2008)	
	Differential pulse voltammetry	total PSA	direct assay on single-walled carbon nanotube array-modified microelectrodes	0.25 ng mL ⁻¹	-	-	(Okuno et al., 2007)	
	Voltammetric enzyme-linked immunoassay	PSA	sandwich assay	-	0.1 ng mL ⁻¹	-	(Zhang et al., 2007b)	

Nomenclature



1.6 AIMS AND OBJECTIVES

PSA test is widely used for prostate cancer diagnosis. However, due to its low specificity and the need to confirm the results with invasive tests, the search for a better test continues. Additionally, the advances of biosensor technology started to provide simple, rapid and multi-analyte testing systems that will revolutionise both the diagnosis and prognosis of diseases and eliminate the patient waiting time for the results. One of the objectives of the study was to develop a new test for prostate cancer diagnosis/prognosis using rapid and sensitive system which can be applied for multi marker detection. Another was to develop new sensor surface chemistries for gold-based biosensor platforms to enhance the sensitivity of these types of devices. In addition, to test the suitability of a capacitive biosensor for prostate cancer testing and its potential for multi-biomarker analysis. In the study four types of sensors were used and these include QCM and SPR instrument prototyped developed by Sierra Sensors GmbH (Hamburg, Germany), a commercial SPR biosensor (Biacore 3000, GE Healthcare, Sweden) and an interdigitated array capacitive biosensor developed by Sabanci University (Turkey).

The work programme carried out is summarised in Figure 1.18 as a flowchart. This included:

- I. Development of sensor surface chemistry protocols and testing for:
 - a. Self assembled monolayer
 - b. Dendrimer surface
 - c. A new polymer surface
 - d. Carboxymethyl dextran surface

II. Development of an affinity assay for PSA testing

- a. Direct and sandwich QCM immunoassay for PSA detection
- b. Development of a new buffer to minimise serum non-specific binding on sensor surface
- c. PSA detection in serum using the new buffer developed with QCM sensor and calibration curve calculation
- d. PSA assay confirmation using a commercial SPR biosensor
- e. Testing patient (Bedford Hospital NHS Trust) and control (Cranfield University staff) serum samples using the developed QCM immunoassay
- f. Testing the feasibility of free-PSA, complexed-PSA and prostate specific membrane antigen (PSMA) immunoassays

III. Capacitive biosensor development for PSA testing

- a. Testing the existing capacitive biosensor developed at Sabanci University
- b. Development of a new capacitive biosensor
- c. Improvements to the fabrication of interdigitated array capacitive biosensor chip

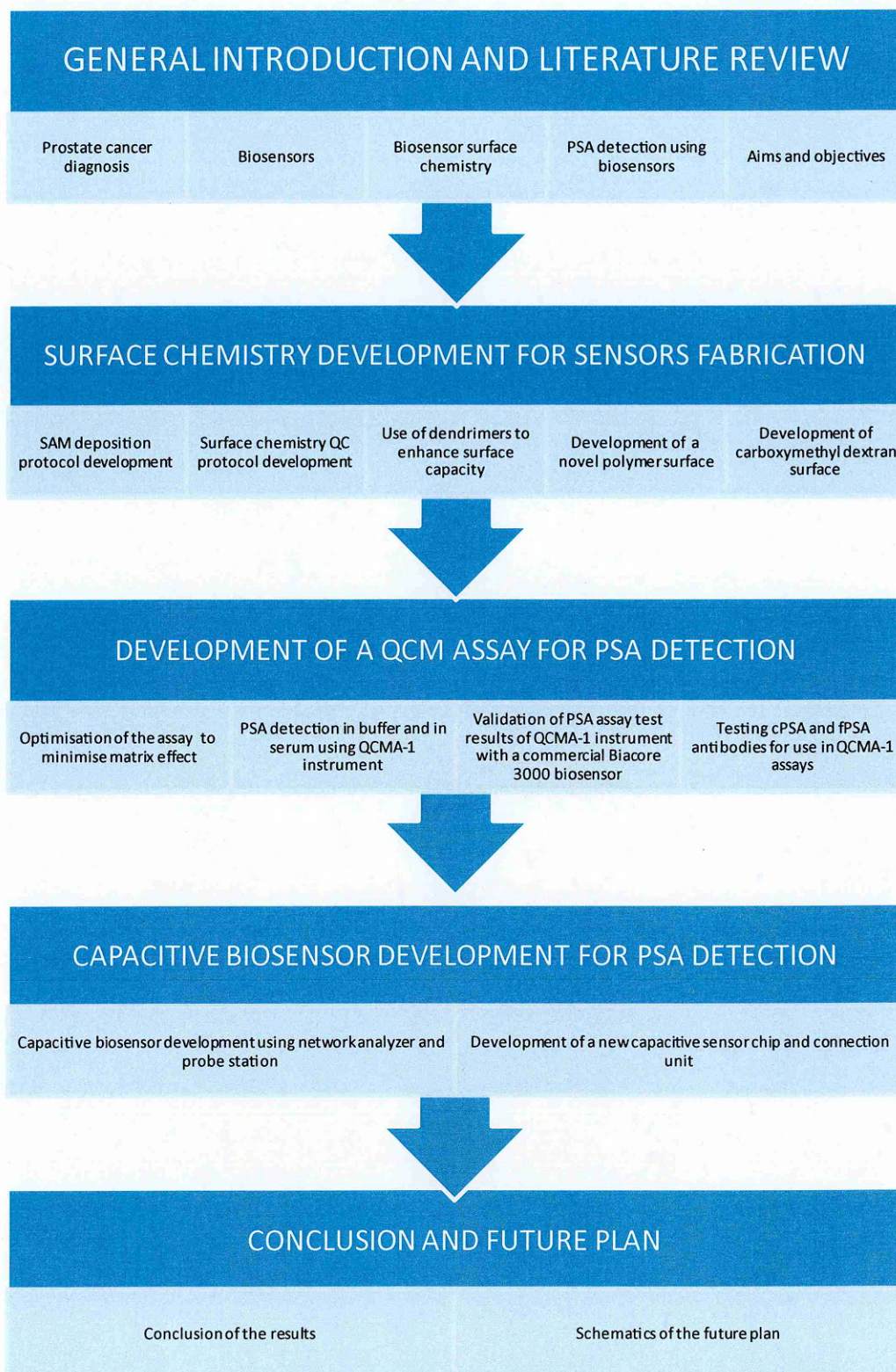


Figure 1.18. Flowchart outlining the phases of work carried out in the study.

CHAPTER II:

SURFACE CHEMISTRY DEVELOPMENT FOR SENSORS

2 SURFACE CHEMISTRY DEVELOPMENT FOR SENSORS

2.1 INTRODUCTION

The type of surface chemistry used to functionalise biosensor chips is crucial to obtain good detection signal from the device. SPR, QCM and electrochemical sensor chips use a metal layer, mostly gold as the working electrode. The direct adsorption of proteins to a metal surface may cause the denaturation of the proteins and also the orientation of the proteins on the metal surface may block the active binding site for ligand binding. Additionally the weak interaction between the surface and the protein will make the sensor unstable for repeat use and the regeneration solutions will cause proteins to be removed from the surface. To eliminate these problems, the use of covalent immobilization is usually recommended in order to have a stable sensing layer covering the metal surface by either a self assembled monolayer or a polymeric surface. This will enable proteins to be immobilized covalently which will result in a stable protein layer that may be used several times without activity loss.

In the current study initially self assembly of alkanethiols were considered to coat the gold surface of the biosensor chip. The self assembly spontaneously occurs due to the strong interaction between the sulphur groups of the alkanethiols and the gold surface (Poirier and Pylant, 1996). Many different alkanethiol molecules with different active end groups are commercially available for monolayer deposition. For example alkanethiols with biotin end group enable preparation of avidin layers to immobilise biotinylated proteins (Chung et al., 2006). Alkanethiols with end groups

of amine and carboxyl are commonly used to immobilise proteins to the sensor surfaces (Navrátilová and Skřidala, 2004; Oh et al., 2004). Mercaptoundecanoic acid (MUDA) is a well known alkanethiol molecule that provides well ordered self assembled monolayer and also is cheaply available. For this reason MUDA was chosen as a surface coating of the QCMA-1, Biacore and the capacitor sensor surfaces. In order to be able to increase the capacity of the sensing layer as an alternative to monolayers; dendrimers, surface grafted polymers and carboxymethyl dextran surfaces were investigated. The assays performed using MUDA coated sensor chips provided a basis to compare and improve the new surface chemistries developed.

2.2 MATERIALS AND INSTRUMENTATION

Phosphate buffered saline (PBS, 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4) tablets, mercaptoundecanoic acid (MUDA), ethanolamine, ethylene glycol dimethacrylate (EDMA), Methacrylic acid (MA), biotinylated-BSA, amino terminated generation 4 polyamidoamine (PAMAM) dendrimer, cysteamine, N,N'-bis(acryloyl)cystamine, acetonitrile, toluene, spectrophotometric grade ethanol and potassium ferricyanide, diethylene glycol dimethyl ether (diglyme), epichlorohydrine, bromoacetic acid, dextran (MW 500 000) and carboxymethyl dextran (MW 10,000 – 20,000) were purchased from Sigma-Aldrich (Poole, UK). Potassium chloride (KCl) was purchased from Fisher Scientific (Loughborough, UK). Dimethyl formamide (DMF) was purchased from Acros Organics (Loughborough, UK) and Diethyldithiocarbamic acid benzyl ester was supplied by TCI Europe (Antwerp, Belgium). 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and NeutrAvidin (NA) and EZ-link-sulfo-NHS-Biotin was purchased from Pierce-Thermo Scientific (Cramlington,

UK). Oxygen free nitrogen was purchased from BOC (Manchester, UK). Mercaptohexadecanol was purchased from Asemblon (Redmond, USA). Ultrapure water ($18\text{ M}\Omega\text{ cm}^{-1}$) was obtained from a Milli-Q water system (Millipore Corp., Tokyo, Japan).

Fully automated QCMA-1, SPR2 (Sierra Sensors GmbH, Germany) and Biacore 3000 (GE Healthcare, Sweden) biosensors and bare gold sensor chips were used for the experiments (Figure 2.1). QCMA-1 (20 MHz) and SPR2 sensor chips possess two sensing array and the Biacore sensor chips possess four sensing array, enabling the measurement of active and control sensor surfaces simultaneously.

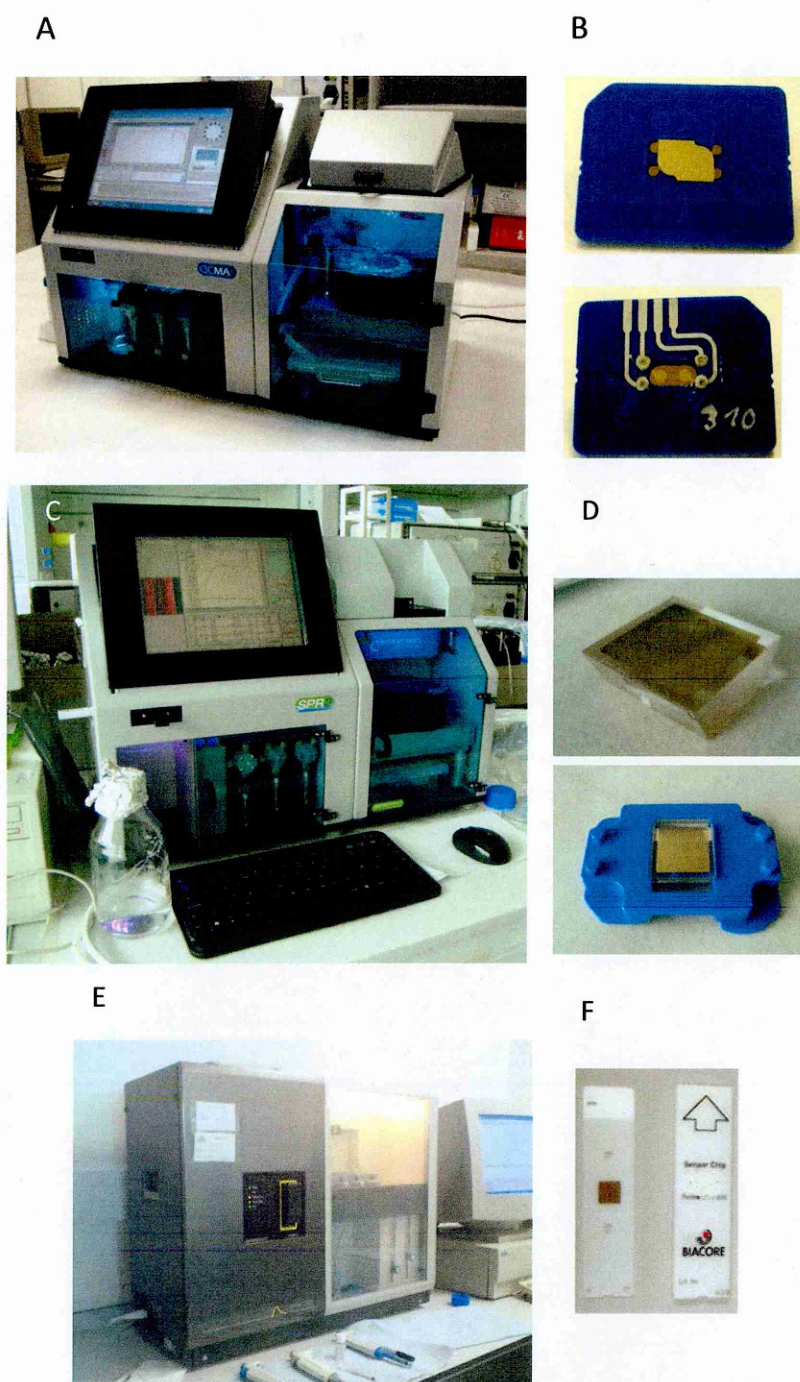


Figure 2.1. QCMA-1 (A) and SPR2 (C) biosensors from Sierra Sensors GmbH (Hamburg, Germany) and Biacore 3000 (E) biosensor from GE Healthcare (Uppsala, Sweden) were used to perform assays. The sensor chips used for the devices are shown at figures B, D and F.

The sensor chip surface cleaning was carried out using an Emitech K 1050X (EM Technologies Ltd., Kent, UK) plasma asher. The refractive index and extinction coefficient of bare gold sensor and the thickness values for polymer coated sensors were obtained using a SE 400 Ellipsometer (Mi-Net Technologies Ltd., Maidenhead, UK). For AFM measurements Dimension 3000 SPM instrument (Veeco Instruments Ltd., Cambridge) was used with silicon probes (type: PPP-NCH-50). For UV polymerisation a Philips HB171/A UV device (220-230V, 50Hz, 75W, UV type 3) and Cermax (Xenon Fiberoptic Lightsource, ILC Technology Inc., 100-120/200-240 V, 50/60Hz) were employed. The contact angle measurements were performed using a KSV CAM 100 Optical Contact Angle Meter (L.O.T. Oriel Ltd., Leatherhead, UK). Cyclic voltammetry measurements were performed with an Autolab Electrochemical Analyzer with the general purpose electrochemical software GPES 4.9 (Ecochemie, Utrecht, Netherlands). A medium sized Atmosbag from Sigma-Aldrich (Poole, UK) was used to obtain argon filled closed environment for UV polymerisation.

2.3 METHODS

2.3.1 Self assembled monolayer deposition

Initially the Petri dish that would be used for SAM deposition was cleaned with nitrogen plasma for one minute using Emitech K 1050X (EM Technologies Ltd., Kent, UK) plasma asher, then rinsed with deionised water and ethanol. Then spectrophotometric grade ethanol was added to the Petri dish and the lid closed. QCMA-1 sensor chips were placed on a Petri dish and plasma cleaned with nitrogen plasma for one minute at different power and pressure settings to optimise the cleaning conditions (40 W and 50 W, 2×10^{-2} mbar and 5×10^{-2} mbar). The sensor

chips were transferred to the SAM deposition Petri dish filled with ethanol. A 2 mM MUDA solution was prepared in spectrophotometric grade ethanol and flushed with argon/nitrogen for around 10 minutes. The ethanol in the Petri dish was removed with help of a pipette or syringe then MUDA solution was added. The Petri dish was covered with parafilm and aluminium foil and incubated overnight at ambient temperature. Next morning the sensor chips were rinsed with ethanol and water and dried with nitrogen stream. The SAM coated sensors were placed in a Petri dish and stored in the fridge till further use.

2.3.2 Contact angle measurement

Static water contact angles were measured with the sessile drop method using a KSV CAM 100 Optical Contact Angle Meter (L.O.T. Oriel Ltd., Leatherhead, UK) at ambient temperature. The water droplets were imaged by a camera before the software calculated their shape and the respective contact angles. The contact angles of three water droplets were measured for each sensor chip and the average of these were used as the resulting contact angle. A sample contact angle measurement to a water droplet on the gold sensor chip is shown in Figure 2.2.

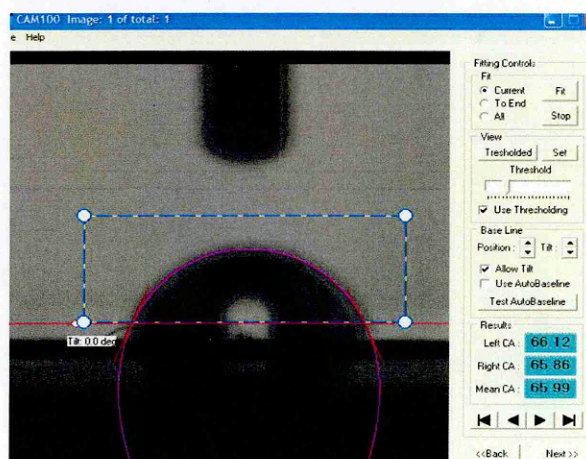


Figure 2.2. Contact angle measurements of a water droplet on the gold sensor chip.

2.3.3 Cyclic voltammetry

Cyclic voltammetry was used to characterize the working electrode surface of the QCM sensor chips. Cyclic voltammetry is a potentiodynamic electrochemical measurement where the potential is continuously changed as a linear function of time. The rate of change of potential with time is referred to as the scan rate (v). A positive potential scan is chosen for the first half cycle during which an anodic current is observed. Because the solution is quiescent, the product generated during the forward scan is available at the surface of the electrode for the reverse scan resulting in a cathodic current. During the potential sweep, the potentiostat measures the current resulting from the applied potential. The resulting plot of current vs. potential is termed a cyclic voltammogram.

Cyclic voltammetry measurements were performed with an Autolab Electrochemical Analyzer with the general purpose electrochemical software GPES 4.9 (Ecochemie, Utrecht, Netherlands). In this study 1 mM potassium ferricyanide prepared in 1M KCl was used as an electroactive marker for cyclic voltammetry measurements. The gold electrode surface of the QCM sensor chip forms the working electrode. After 80 μ l of potassium ferrocyanide solution was placed on the horizontally kept electrode surface, reference (Ag/AgCl) and counter (carbon) electrodes were placed from the top till they touch the solution (Figure 2.3).

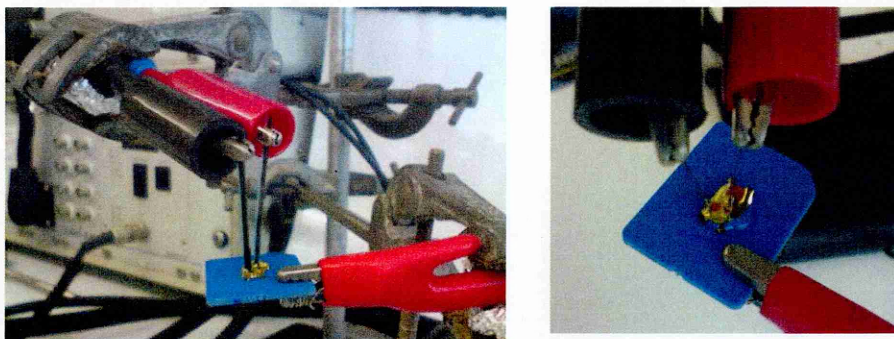


Figure 2.3. Cyclic voltammetry set up for QCM sensor chips.

Scan for potassium ferricyanide was set to 3 cycles from 0 to 0.5 V relative to Ag / AgCl reference electrode and carbon counter electrode. The summary of cyclic voltammetry parameters are listed in Table 2.1.

Table 2.1. The parameters of cyclic voltammetry measurements.

Cyclic voltammetry parameters	
Electroactive marker:	1 mM potassium ferricyanide in 1 M KCl
Working electrode:	Au
Reference electrode:	Ag / AgCl
Counter electrode:	Carbon
Step potential:	0.00198 V
Potential range:	0 – 0.5 mV
Scan rate:	0.05 V/s
Number of scans:	3

The surface coverage (θ) of the self assembled monolayer can be calculated by comparing the charge (Q) during reduction/oxidation of cyclic voltammetry at a monolayer covered electrode with respect to a bare gold electrode (Equation 2.1)(Campuzano et al., 2006; Yang and Khoo, 2004).

$$\theta = 1 - \frac{Q_{SAM}}{Q_{bare\ gold}} \quad (\text{Eq. 2.1})$$

The area under the peak of cyclic voltammogram gives the charge (Q) created during cyclic voltammetry (Wang, 2006). To calculate the surface coverage of the SAM, first cyclic voltammograms of bare gold and SAM coated sensors were transferred to Microsoft Excel and Trapezoid rule was used to calculate the area between oxidation and reduction curves to obtain the charges $Q_{bare\ gold}$ and Q_{SAM} (Liengme, 2008). Then Equation 2.1 was used to obtain surface coverage of SAM coated sensor chips.

2.3.4 Atomic Force Microscopy Analysis

Atomic Force Microscope (AFM) consists of a micro-cantilever tip that is used to scan an area to create the image of a surface. For the study Dimension 3000 SPM instrument (Veeco Instruments Ltd., Cambridge) was used with silicon probes (type: PPP-NCH-50). The analysis was carried out at tapping mode. Prior to the analysis sensor chips were plasma cleaned, rinsed with isopropyl alcohol and dried with pressurised air.

2.3.5 PAMAM dendrimer surface fabrication

Two different methods were considered for surface modification using PAMAM dendrimers. Biotin modified dendrimers were formed on either 10% or 1% MUDA monolayers and the results of NeutrAvidin capture was compared with the surfaces that are MUDA or biotin modified. The aim was to observe if dendrimer modified surfaces provide higher NeutrAvidin capture capacity than the other SAM coated surfaces. The fabrication of dendrimer layers and the surfaces used to compare is described below:

Method 1 - Capture of NeutrAvidin on to biotinylated dendrimer immobilised 10% or 1% MUDA surface: Initially sensor chips were coated with 10% (or 1%) MUDA and 90% (or 99%) mercaptoundecanol using the protocol described in section 2.3.1. The sensor chip was then docked to the QCMA-1 or Biacore 3000 instrument and primed with degassed Dulbecco's modified phosphate buffered saline (PBS) that was used as running buffer. Sensor surface was first activated with a 1:1 mixture of 400 mM EDC and 100 mM NHS (final concentrations: 200 mM EDC and 50 mM NHS). EDC-NHS was injected to sensing spot 3 min. This was followed by two injections of 5% amino dendrimer (MW: 7332) for 5 and 2 minutes, respectively. Then 1M ethanolamine was injected for 3 minutes to the sensor surface to block the remaining active sites (Figure 2.4 - A). To modify the amino terminal groups of the dendrimer with biotin, sulfo-NHS-biotin was injected twice over to sensing spot for 5 and 3 minutes (Figure 2.4 - B). Now the sensor surface was biotinylated and ready for NeutrAvidin capture. As the sensor surface biotinylation was complete, the sensor chip was docked to the biosensor. After priming with PBS, a 100 $\mu\text{g mL}^{-1}$ NeutrAvidin was injected for 3 minutes to the sensor surfaces (Figure 2.5). For some of the Biacore sensor chips amino dendrimer immobilisation and surface biotinylated was performed static in a Petri dish.

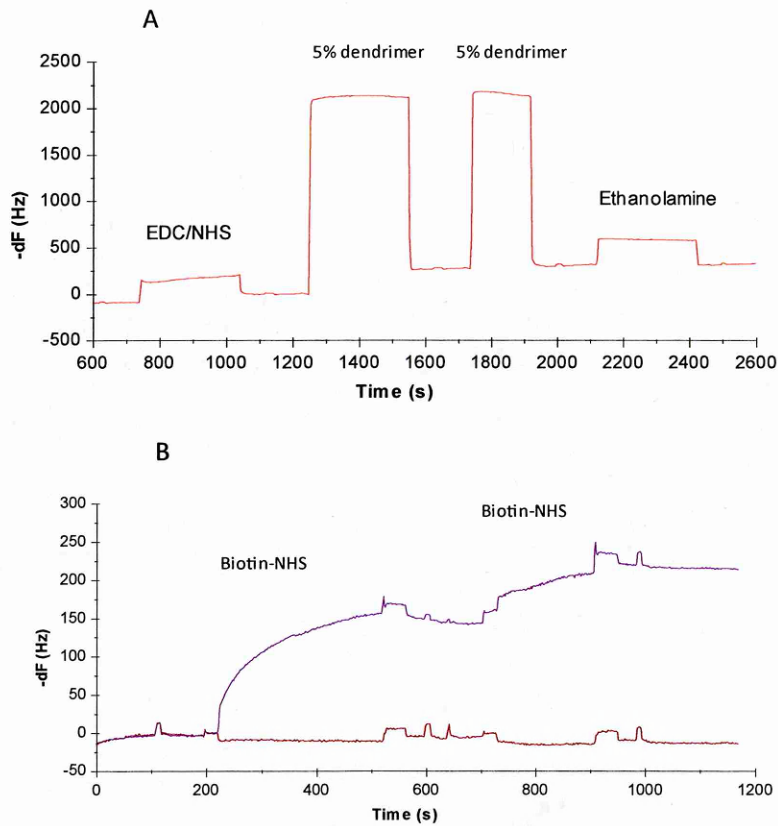


Figure 2.4. (A) Immobilisation of amino dendrimer on to the 10% MUDA modified QCMA-1 sensor chip (305 Hz). (B) Immobilisation of sulfo-NHS-biotin on the amino terminal groups of dendrimers that were immobilised to the QCMA-1 sensor chips (215 Hz).

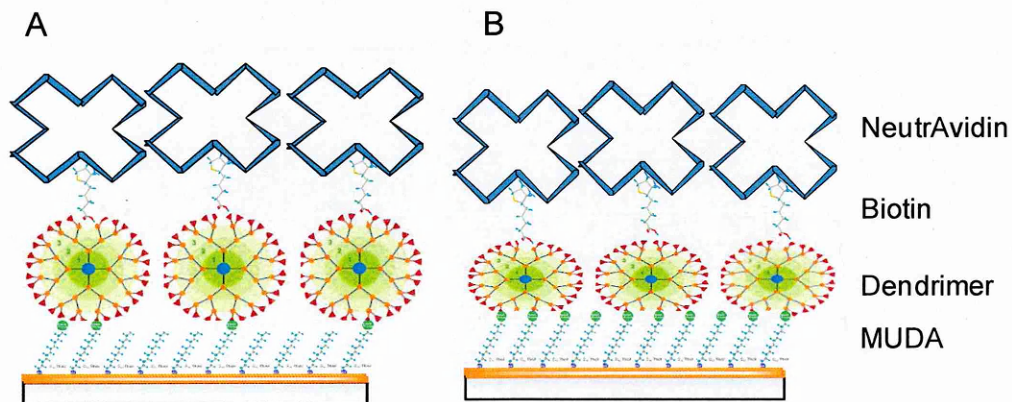


Figure 2.5. Schematics of NeutrAvidin captured surface prepared by biotinylated dendrimer monolayer on 1% (A) and 10% (B) MUDA coated sensor chip.

Method 2 - Immobilisation of NeutrAvidin on to 10% MUDA coated sensor surface:

The immobilisation was achieved using conventional amine coupling chemistry on MUDA coated sensor chips (Staros, Wright and Swingle, 1986). Running buffer used for immobilisation was degassed Dulbecco's modified phosphate buffered saline (PBS). Sensor surfaces were first activated with a 1 : 1 mixture of 400 mM EDC and 100 mM NHS, prepared in 0.22 μm filtered deionised water, and mixed immediately prior to use (final concentrations: 200 mM EDC and 50 mM NHS). EDC– NHS was injected simultaneously across the sensor surface for 3 min. A 100 $\mu\text{g mL}^{-1}$ NeutrAvidin was injected for 3 minutes. The sensor surface was then blocked with 1 M ethanolamine, pH 8.5 for 3 minutes. The frequency changes were recorded 2 minutes after the protein injection was completed (Figure 2.6).

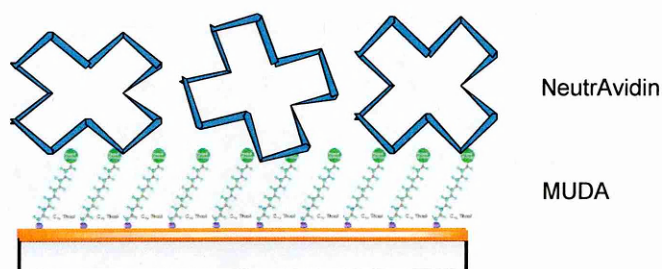


Figure 2.6. Schematics of NeutrAvidin immobilised MUDA coated sensor chip.

Method 3 - Capture of NeutrAvidin on to biotinylated cysteamine ($\text{SH-C}_2\text{H}_4\text{-NH}_2$) surface (Figure 2.7): Initially the sensor surface was modified with a methanolic solution of 5mM cysteamine using the SAM deposition protocol described in section 2.3.1. Later 2.5 mg EZ link-Biotin-LC-NHS was dissolved in 200 μl PBS and added to the sensor chips in a Petri dish for 2 hours incubation at 25 $^{\circ}\text{C}$. Then the sensor chips were washed with PBS and deionised water several times and dried with a nitrogen stream. As the sensor surface biotinylation was complete, the sensor chip

was docked to the biosensor. After priming with PBS, a $100 \mu\text{g mL}^{-1}$ NeutrAvidin was injected for 3 minutes to the sensor surfaces.

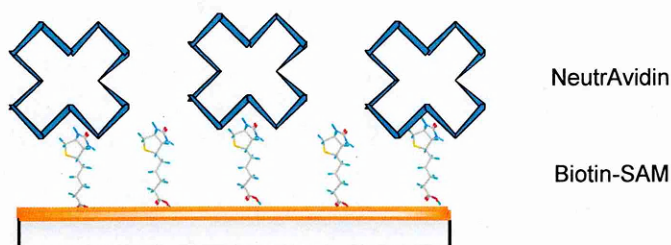


Figure 2.7. Schematics of NeutrAvidin capture on biotin modified sensor surface.

2.3.6 UVlink polymer coating on sensor surfaces

Initially the sensor chips were coated with N,N'-bis(acryloyl)cystamine (BAC). Au coated QCMA-1 sensor chips were cleaned using a plasma etching process for 1 minute under nitrogen. The cleaned sensor chips were then submerged in methanolic solution of 2.5 mM BAC overnight (Figure 2.8). After rinsing with methanol and water, the sensor chips were dried with a nitrogen stream.

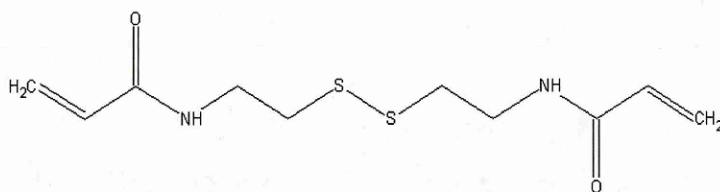


Figure 2.8. The chemical structure of N,N'-bis(acryloyl)cystamine.

The polymerization was carried out in organic solution using toluene and water as solvents, MA as functional monomer, and EDMA as the cross-linking monomer. An initiator comprising diethyldithiocarbamic acid benzyl ester was used for the polymerisation (Figure 2.9).

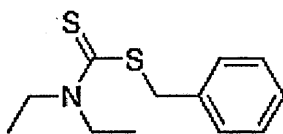


Figure 2.9. The chemical structure of diethyldithiocarbamic acid benzyl ester.

The polymerization was carried out in organic solution using toluene and water as solvents, MA as functional monomer, and EDMA as the cross-linking monomer. An initiator comprising diethyldithiocarbamic acid benzyl ester was used for the polymerisation. Both the initiator and the polymerisation solutions were sonicated (15 min) and then purged with nitrogen (10 min) to reduce the amount of oxygen in the solution prior to polymerization and also disperse the water in toluene. In some cases polymerization mixture was sonicated for further 30s just prior to polymerization. Polymerisation was carried out at room temperature, in an Atmosbag to create an oxygen free environment. The Atmosbag was flushed with argon at all times. Initially sensor surfaces were placed in a Petri dish filled with initiator (10 mM in acetonitril) and UV polymerized for 20 minutes. The Petri dish was removed from the Atmosbag and sensor chips were rinsed with the solvent 3 times, then rinsed with methanol and dried before the addition of polymerisation mixture comprising crosslinker and monomer. The sensor chips were UV irradiated at varying times (20 to 90 minutes); then rinsed with toluene, methanol and finally with deionized water before drying with a nitrogen stream. The UVlink polymer coated sensor chips were stored at room temperature for next day testing (Figure 2.10). Some sensor chips were treated with methanol overnight before drying to increase the stiffness of the polymer created (Kitagawa, 1999).

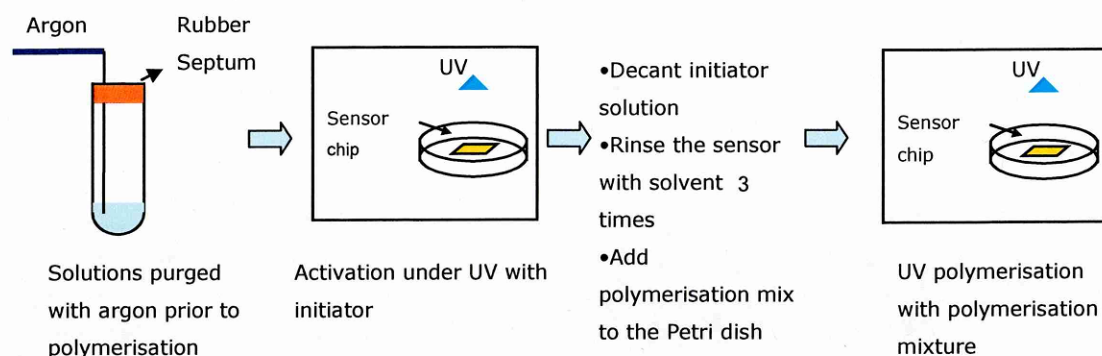


Figure 2.10. Experimental set up for UVlink polymer coating.

2.3.7 Preparation of carboxymethyl dextran surface

Two common methods are reported in the literature to achieve CMD coated sensor surface and both methods were tested in the current study:

Amide-linked CMD: Fabricating an amino end group SAM on the sensor surface and attachment of CMD with conventional EDC/NHS chemistry (Jung et al., 2007). If the CMD is obtained ready-made, this method is simple, contains less steps, less chemicals and short.

A. Amide-linked CMD fabrication in-line in the instrument

After plasma cleaning bare gold Biacore sensor chips were incubated in 10% mercaptoundecanoic acid (MUDA) overnight (10%, 2 mM mercaptoundecanoic acid and 90%, 2 mM mercaptoundecanol). Sensor chips were then washed with ethanol and deionised water and dried with a nitrogen stream. The SAM coated sensor was first docked to the Biacore instrument and primed with phosphate saline buffer (pH 7.4). The flow rate of the instrument was set to $10 \mu\text{l min}^{-1}$. The assay was then

preceded as follows: A 3 minutes injection of 1:1 mixture of 400 mM 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and 100 mM N-hydroxysuccinimide (NHS). A 5 minutes injection of 200 mM ethylene diamine. NHS-dextran was prepared by mixing 240 μ l 100 mM NHS, 240 μ l 400 mM EDC and 480 μ l 0.5 g mL⁻¹ CMD (Fluka, cat no: 86524, MW 10 000 - 20 000) in PBS and incubated for 5 minutes at room temperature. NHS-dextran was injected to the sensor surfaces for 2 x 5 minutes and 3 minutes. The surface was then washed with 0.1 M HCl for 2 x 1 minute.

B. Amide-linked dextran fabrication in static conditions

After plasma cleaning bare gold sensor chips were incubated in 10 % mercaptoundecanoic acid (MUDA) overnight. (10%, 2 mM mercaptoundecanoic acid and 90%, 2 mM mercaptoundecanol). The sensor chips were then washed with ethanol and deionised water and dried with a nitrogen stream. EDC/NHS was placed as a droplet on the sensor surfaces (270 μ l on Biacore chips, 400 μ l on SPR-2 chips) and incubated for 5 minutes. Sensor chips were then washed with deionised water 3 times. The sensor chips were placed on a Petri dish containing 500 mM ethylene diamine and incubated for 30 minutes before washing with deionised water 5 times. A 1 g of CMD was dissolved in 1 mL of PBS; 600 μ l EDC and 600 μ l NHS were added to the solution and incubated for 5 minutes at room temperature. CMD/EDC/NHS mixture (NHS-dextran) was placed on each sensor chip (270 μ l on Biacore chips, 400 μ l on SPR-2 chips) and incubated for 2 hours at room temperature. Later the sensor chips were washed 5 times with deionised water. Then the sensor chips were left in water for 5 minutes and washed again with deionised water at 50 °C for 10 times. The number of carboxyl groups were increased by application of bromoacetic acid (1 M bromoacetic acid in 2 M sodium hydroxide at

25 °C for 4 hours. Then the sensor chips were washed 5 times with deionised water at 50 °C. Sodium salt modified carboxyl groups of the dextran was reactivated again by application of bromoacetic acid (1 M bromoacetic acid in 2 M sodium hydroxide at 25 °C for 16 hours. Later the sensor chips were washed 5 times with deionised water. Then the sensor chips were left in water for 5 minutes and washed again with deionised water at 50 °C for 10 times. CMD modified sensor chips were dried with a nitrogen stream and kept in the fridge for further use.

Epoxy-linked CMD fabrication procedure

Fabricating a hydroxyl end group for SAM modification on the sensor surface and attachment of dextran using epichlorohydrine. Modification of the hydroxyl groups to carboxyl groups was then conducted by bromoacetic acid. This method was first described in 1990 by Lofas and Johnsson and later patented (Bergstrom, Lofas and Johnsson, 1995; Bergstrom, Lofas and Johnsson, 1993; Lofas and Johnsson, 1990a), it takes around 3 days to produce the sensor chips. After plasma cleaning bare gold sensor chips were incubated in 1 mM mercaptohexadecanol overnight. Later sensor chips were washed with ethanol and deionised water several times and dried with a nitrogen stream. A 3 g of dextran (Sigma, cat no: 31392, MW 500 000) is added to 10 mL of 0.1 M NaOH and mixed and placed on a shaker for 4 hrs and later sonicated for 1 minute to remove the air bubbles from the dextran solution. The sensor chip surface was activated with 0.6 M epichlorohydrin (4.73 mL diglyme + 4.73 mL 0.4 M NaOH + 0.53 mL epichlorohydrin) for 4 hours at 25 °C. Sensor chips were washed with deionised water and dried with a nitrogen stream. The activated sensor chips were treated with the dextran solution in a Petri Dish for 20 hours at 25 °C. Later the sensor chips were washed 5 times with deionised water. Then the sensor

chips were left in water for 5 minutes and washed again with deionised water at 50 °C for 15 times. The dextran coated sensor chips were functionalised by application of bromoacetic acid (1 M bromoacetic acid in 2 M sodium hydroxide) at 25 °C for 4 hours for *method 1* and for 16 hrs for *method 2*. Then the sensor chips were washed 10 times with deionised water at 50 °C. The dextran coated sensor chips were functionalised again by application of bromoacetic acid (1 M bromoacetic acid in 2 M sodium hydroxide) at 25 °C for 16 hours. Later the sensor chips were washed 10 times with deionised water. Then the sensor chips were left in water for 5 minutes and washed again with deionised water at 50 °C for 10 times. CMD modified sensor chips were dried with a nitrogen stream and kept in the fridge for further use.

2.3.8 Testing of the developed polymer surfaces

Functional assay using the surface grafted sensor chips were performed using QCMA-1, SPR2 or Biacore 3000 instruments. The operating temperature of the assays was 25 °C and the flow rate of the buffer was 80 $\mu\text{l min}^{-1}$ for QCMA-1, 25 $\mu\text{l min}^{-1}$ for SPR2 and 10 $\mu\text{l min}^{-1}$ for Biacore 3000 assays. The polymer coated sensor was first docked to the instrument and primed with phosphate saline buffer (pH 7.4). Rabbit anti-mouse IgG and Mouse IgG antibodies were then immobilised using conventional amine coupling chemistry. Sensor surfaces were first activated with a 1:1 mixture of 400 mM 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and 100 mM N-hydroxysuccinimide (NHS), prepared in deionised water, and mixed immediately prior to use (final concentrations: 200 mM EDC and 50 mM NHS). EDC-NHS was injected simultaneously across the four sensing spots for 3 min (150 μl). A 100 $\mu\text{g mL}^{-1}$ rabbit anti-mouse IgG and 100 $\mu\text{g mL}^{-1}$ mouse IgG (both in sodium acetate buffer, pH 5.5) were injected on the two sensing arrays for 3 min

(150 μ l). Non-reacted NHS esters were capped with 1 M ethanolamine, pH 8.5 for 3 min (150 μ l). The response changes were recorded 180s after the injection started (as antibody capture response) and 2 min after the protein injection was completed and the signal reached steady state (as the immobilisation response).

After antibody immobilisation, 5 μ g mL⁻¹ mouse IgG (in PBS) was injected over rabbit anti-mouse-IgG and mouse IgG immobilised sensing arrays for 3 minutes to allow binding. The frequency changes due to mouse IgG binding were recorded 180 s after the injection started. After a dissociation period under running buffer flow (180s), surfaces were regenerated by injection of 100 mM HCl (60s).

2.4 RESULTS AND DISCUSSION

2.4.1 Self assembled monolayer deposition

Before the application of SAM deposition, it is essential to clean the sensor surface adequately to enable perfect surface coverage of the monolayer, for this purpose a cleaning protocol was optimised. The effectiveness of the cleaning method was tested using contact angle measurements and AFM analysis.

SAMs can be prepared simply by adding a solution of the desired alkanethiol in ethanol onto the gold sensor chip and after the incubation time the excess can be washed off. The protocol of alkanethiol self assembly on gold substrates is well documented in the literature as dilute SAM solution (1 - 5 mM) and long incubation period (15 - 24 hrs) is required for the formation of a compact monolayer (Asemblon, 2005b; Bard and Rubinstein, 1996). Therefore in this study an overnight incubation with 2 mM ethanolic MUDA solution was used for SAM deposition and no further optimisation was performed and the quality control of the sensor chips proved the compactness of the formed monolayer. The quality control checks on the alkanethiol coated sensor chips can be performed using optical (such as contact angle, AFM or SEM measurements) and electrochemical methods (such as cyclic voltammetry or impedance analysis). In this study cyclic voltammetry was used to characterise the MUDA coated sensor surfaces. It is not possible to conduct cyclic voltammetry measurements to the Biacore sensor chips with the current set up available. Therefore the surface chemistry optimisation was performed using QCMA-1 sensor chips.

2.4.1.1 Contact angle measurement

If a freshly evaporated gold substrate is used for SAM deposition no cleaning is needed, otherwise to remove adsorbed hydrocarbons and other impurities a pre-treatment method needs to be applied. The pre-treatment may consist of electrochemical cleaning (electrochemical cycling with dilute acids), chemical cleaning (piranha cleaning, strong acid/base treatment, sonication in alcohol), plasma cleaning (mainly argon and oxygen) or UV/ozone irradiation (Asemblon, 2005b; Bard and Rubinstein, 1996). For the cleaning of gold sensor surfaces two approaches were considered as ethanol wash and plasma cleaning. “The wetting properties of bare gold serve as a sensitive test for the presence of organic contaminants, any partial dewetting of the gold surface by water indicates incomplete cleaning (Bard and Rubinstein, 1996).” The water contact angle measurements were performed on the sensor surfaces after each treatment to investigate the hydrophobicity of the sensors to compare the effect of the gold cleaning methods used.

The contact angle of a water droplet was measured on bare gold QCMA-1 sensor chips, after ethanol wash and after plasma cleaning. As it could be seen from Figure 2.11, there was little difference in contact angles before and after the cleaning of the gold sensor chips with ethanol. However, as plasma cleaning was applied there was a dramatic decrease on water contact angle up to ca. 14 degrees (after plasma cleaning at 50 W power and 5×10^{-2} mbar pressure) indicating that the surface impurities were removed with optimised plasma cleaning procedure and a clean and hydrophilic gold surface was obtained. As a result nitrogen plasma cleaning (50 W, 5×10^{-2} mbar pressure) was chosen as the preferred method for cleaning sensor chips prior to SAM deposition.

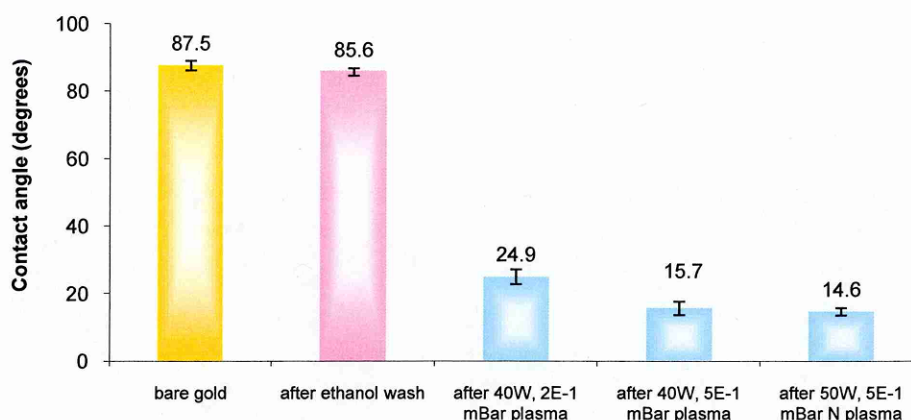


Figure 2.11. Contact angle measurements of gold sensor chips before and after cleaning ($n = 2$ or 4).

An example to the optimisation of substrate plasma cleaning protocol is the study by Raiber *et al.* where they used hydrogen and oxygen plasma to obtain a clean gold surface for SAM application (Raiber *et al.*, 2005). While they obtained 0 degrees contact angle after 20s of oxygen plasma, 70 degrees was obtained after hydrogen plasma cleaning process. The authors' comment that, the unexpected high contact angle of hydrogen plasma could be due to the attraction of hydrocarbons to the clean surface and instantly increasing the contact angle and this phenomenon has been seen at other studies as well. In the case of oxygen plasma, an oxide layer on the gold forms and this layer quickly oxidizes hydrocarbons and keeps the surface hydrophilic. To obtain zero degrees contact angle after cleaning, it is essential to keep the gold substrate away from the ambient air and perform the measurements as quick as possible. In another study, Min *et al.* compared three different cleaning procedures; super-piranha cleaning [(1:10:6) 61% HNO_3 : 30% H_2O_2 : 95% H_2SO_4 (v/v/v)], piranha cleaning [(1:4) 30% H_2O_2 : 98% H_2SO_4 (v/v/v)] and 5 minutes sonication of gold substrates in ethanol (Min *et al.*, 2008). The contact angle

measurements showed that the lowest contact angle measurement was obtained as 60 degrees when super-piranha cleaning method was utilised. High contact angle after super-piranha cleaning indicated that cleaning performance of piranha or super-piranha cleaning process was not better than plasma cleaning that was used in this study. Care needs to be taken while using piranha solution as it is very reactive and dangerous. And also excess rinsing of the substrates is needed to remove any remaining piranha solution prior to SAM deposition. With respect to piranha cleaning or any other chemical cleaning methods, plasma cleaning is simple, easy and use of dangerous chemicals are not required. Tsai *et al.* compared three different cleaning procedures; O₂ plasma cleaning, O₂ plasma cleaning followed with ethanol rinse and O₂ plasma cleaning followed with 1 hour sonication in ethanol; and the contact angle of water obtained using these methods were $9.3^\circ \pm 2.8^\circ$, $18.8^\circ \pm 2.5^\circ$ and $77.9^\circ \pm 10.7^\circ$, respectively (Tsai and Lin, 2001). Low contact angles obtained using the first two methods showed the effectiveness of the oxygen plasma cleaning method. However, oxygen plasma creates a gold oxide layer on the substrate and this layer needs to be reduced completely with ethanol prior to SAM deposition.

In conclusion, the obtained water contact angle of ca. 14 degrees after nitrogen plasma cleaning indicated that the gold sensor surface was clean enough to apply SAM deposition and the result is comparable with the studies in the literature. Later, cyclic voltammetry method was used to assess the quality of the formed MUDA monolayer after nitrogen plasma cleaning.

2.4.1.2 Cyclic Voltammetry

Application of SAM on a gold electrode surface insulates the electrode surface. By comparing the cyclic voltammograms of a bare gold electrode and alkanethiol coated

gold electrode, it is possible to investigate the coverage of the monolayer on the gold electrode. Cyclic voltammograms of bare gold and MUDA coated QCMA-1 sensor chips were obtained using potassium ferricyanide as the electroactive marker. As it could be seen from Figure 2.12, the oxidation and reduction peaks of potassium ferricyanide was visible and there was a larger area between positive and negative potential sweeps when cyclic voltammetry performed on bare gold sensor chip. Whereas, the area between positive and negative potential sweeps of a MUDA coated sensor chip was very small. This indicated the formation of an insulating MUDA monolayer on the sensor surface.

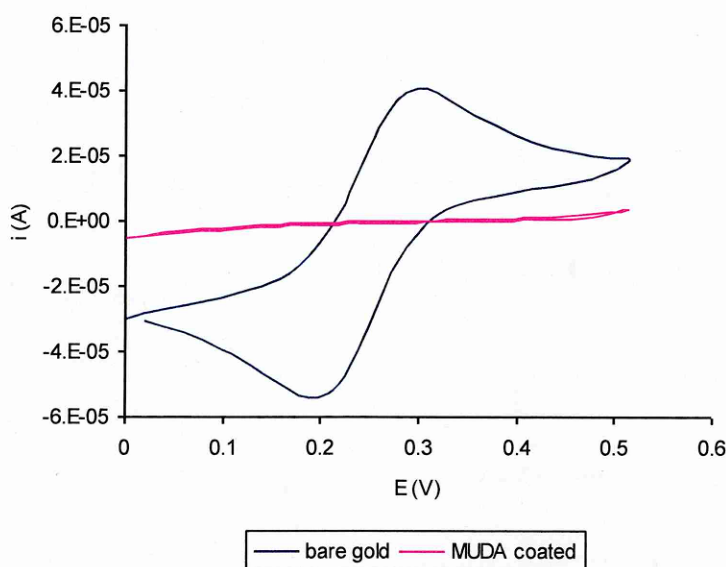


Figure 2.12. Cyclic voltammogram of bare gold and MUDA coated QCM electrodes.

Cyclic voltammetry is a common technique to characterise SAM modified sensor chips (Cavallini et al., 1999; Garc'a-Raya et al., 2008; Shervedani and Bagherzadeh, 2008). When an electrode is modified with SAM, insulating properties of the monolayer causes the peak current to decrease. If the alkanethiol molecule used for

self assembly contains 10 or more alkyl chains (as in the case of MUDA that is used in this study), a very compact insulating layer is formed on the electrode surface and hence the cyclic voltammogram adopts a sigmoidal line shape as microelectrodes. The reason of this microelectrode resemblance of the cyclic voltammogram is the imperfections of the monolayer (pinholes) where electron transfer reaction occurs (Finklea et al., 1993; Janek, Fawcett and Ulman, 1998). Cyclic voltammogram of an MUDA coated QCMA-1 sensor chip showed the same phenomenon, proving that a compact and stable monolayer was formed on the sensor chip surface.

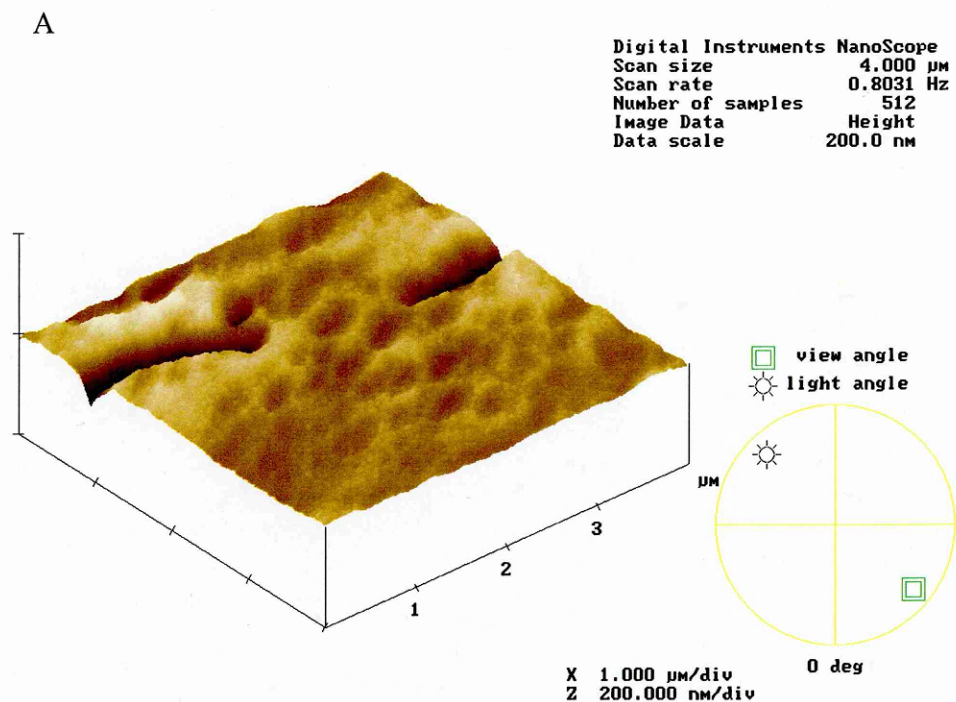
To quantify the surface coverage of the MUDA on the electrode surface, the ratio of the charge obtained during cyclic voltammetry for bare gold and monolayer coated sensors were compared (Equation 2.1, section 2.3.3). The average surface coverage of MUDA coated QCMA-1 sensors were calculated as 0.971 ± 0.015 ($n=7$) indicating that SAM was very compact and the optimised gold cleaning procedure and monolayer deposition technique was efficient. The surface coverage of an electrode is linked to the electrode area. If smaller area is used, it is less likely to find the irregularities and gaps (pinholes) between monolayer patches and this increase the surface coverage. For example, Campuzano *et al.* used a gold disk electrode that has 0.0585 cm^2 geometrical area and calculated surface coverage as 0.997 for an MUDA coated electrode (Campuzano et al., 2006). In our study the electrode area of QCMA-1 sensor chips were ca. 0.42 cm^2 and the obtained surface coverage was 0.971, indicating a very compact MUDA monolayer with respect to the electrode area used for cyclic voltammetry. After each SAM deposition process of the QCM

sensor chips, cyclic voltammetry was used as a quality control method to determine the compactness of the surface coating.

2.4.1.3 AFM Analysis

Plasma cleaning of the sensor chips may etch the gold sensor surface and may affect the surface roughness. Using AFM images the surface roughness of a new and re-used sensor chips were compared. Re-used chip comprises a sensor chip that has been plasma cleaned, SAM deposited and used for QCMA-1 assay 3 times. Both new and re-used chips were cleaned with nitrogen plasma as described at Section 2.3.1, rinsed with isopropanol and dried with an air can prior to obtain AFM images. The AFM images for the new chip are shown in Figure 2.13 and the images for the re-used chip are shown in Figure 2.14.

As seen from the images, deep grooves were observed on both of the sensor chips that are 52.7 nm and 38.4 nm deep. These grooves could be the result of the imperfections of the quartz crystals. In terms of average roughness, there is no significant difference found between the new and 3 times used sensor chip (Table 2.2). This means that the plasma conditions employed for the cleaning of the sensor chip is mild and does not damage the sensor chips even after 3-4 plasma cleaning processes and re-use of the sensor. However the AFM image of the re-used chip showed some white dots indicating that surface is not as clean as the new chip. Therefore it is decided not to use sensor chips more than 3 times as remaining impurities prevent the formation of a perfect SAM a fourth time.



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B

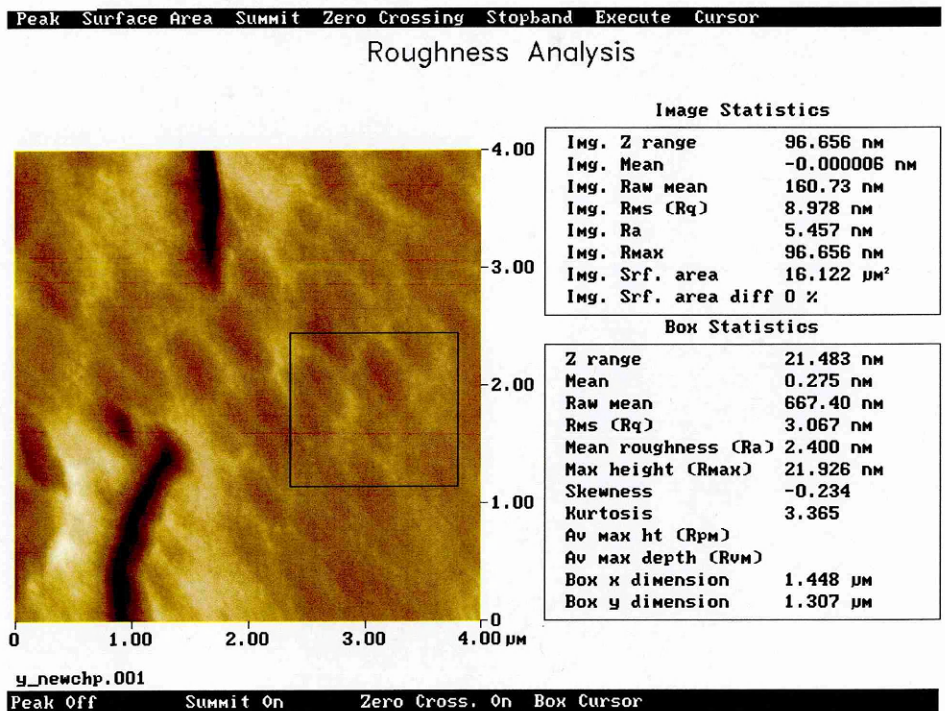


Figure 2.13. AFM image (A) and roughness analysis (B) of a new QCMA-1 sensor chip.

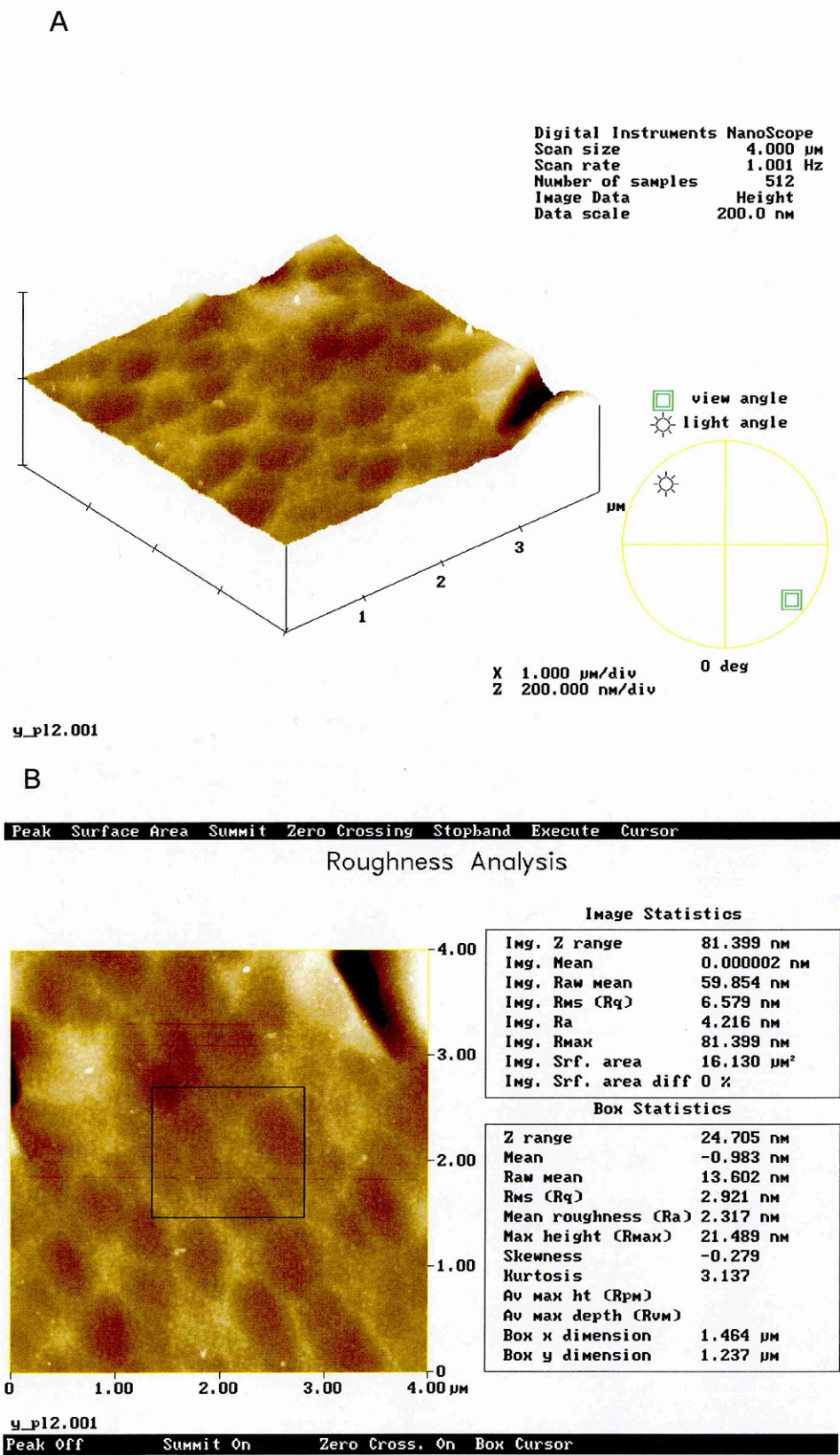


Figure 2.14. AFM analysis of a re-used QCMA-1 sensor chip.

Table 2.2. The results of the AFM analysis on the new and re-used QCMA-1 chips.

		New chip	3 times used chip
Image size 4 μm -4 μm	Mean roughness (nm)	5.46	4.22
	Max height (nm)	96.66	81.40
Image size 1.5 μm - 1.3 μm	Mean roughness (nm)	2.40	2.32
	Max height (nm)	21.93	21.49

2.4.2 Use of PAMAM Dendrimers to enhance surface capacity

In this section the use of amino dendrimers as surface coating is described. Initial idea was to convert the amino terminal groups of the dendrimers to carboxyl groups after dendrimers are immobilised to the MUDA coated sensor chip. However, this process requires the incubation of the sensor chips with succinic anhydride in DMF. It was found that DMF damages the silver paint that was required to connect the electrodes on the QCMA-1 sensor chip to the substrate and also if the treatment was long it damaged the surface of the substrate. Therefore, an alternative method was considered to assess if dendrimer monolayer increases the sensor surface capacity. The alternative method involved the modification of the amino terminal groups of the dendrimer to biotin groups and capture of NeutrAvidin. It is also possible to use a homobifunctional linker that binds the amino group of the proteins to the dendrimer terminal groups, however as the chemicals for biotinylation reagent and NeutrAvidin was readily available for another experiment; biotin-NeutrAvidin route was taken instead of waiting for the new order. The capture capacity of the biotin modified dendrimers was compared to flat surfaces NeutrAvidin immobilisation/capture capacities on MUDA surface and biotin surfaces.

2.4.2.1 Testing of PAMAM amino dendrimer surface for Biacore sensor chips

Initially, bare gold Biacore 3000 sensor chips were modified with 10% MUDA, 1% MUDA and cysteamine (methods 1 and 3 – Section 2.3.2). Amino terminated generation 4 PAMAM dendrimers were immobilised on to MUDA coated Biacore sensor chips in static format. Subsequently cysteamine coated and dendrimer immobilised sensor chips were modified with Sulfo-NHS-Biotin. Later sensor chips were docked one by one to the instrument and the capture of NeutrAvidin was

performed as two 3 minutes injections of $100\text{ }\mu\text{g mL}^{-1}$ NeutrAvidin. The highest NeutrAvidin capture was observed when sensor chips modified with dendrimer that are immobilised on 1% MUDA, the NeutrAvidin capture response was 1.5 times higher than the response obtained from a flat biotin surface (Figure 2.15).

As seen from the Table 2.3 the capture of NeutrAvidin on biotin-dendrimer layer that were immobilised on 10% MUDA gave 1.3 times higher response than NeutrAvidin captured on a flat biotin surface. The NeutrAvidin capture response becomes 1.5 times if a biotin-dendrimer layer that were immobilised on 1% MUDA was used. This result clearly shows that use of dendrimers increase the surface area of the sensor chips to a degree so that more NeutrAvidin capture occurs with respect to a flat surface. Surface area increases with the use of 1% MUDA surface as expected, since less amount of amino terminal groups of the dendrimers were used for the immobilisation to the MUDA surface and hence the shape of the dendrimers are not flattened as seen from Figure 2.16.

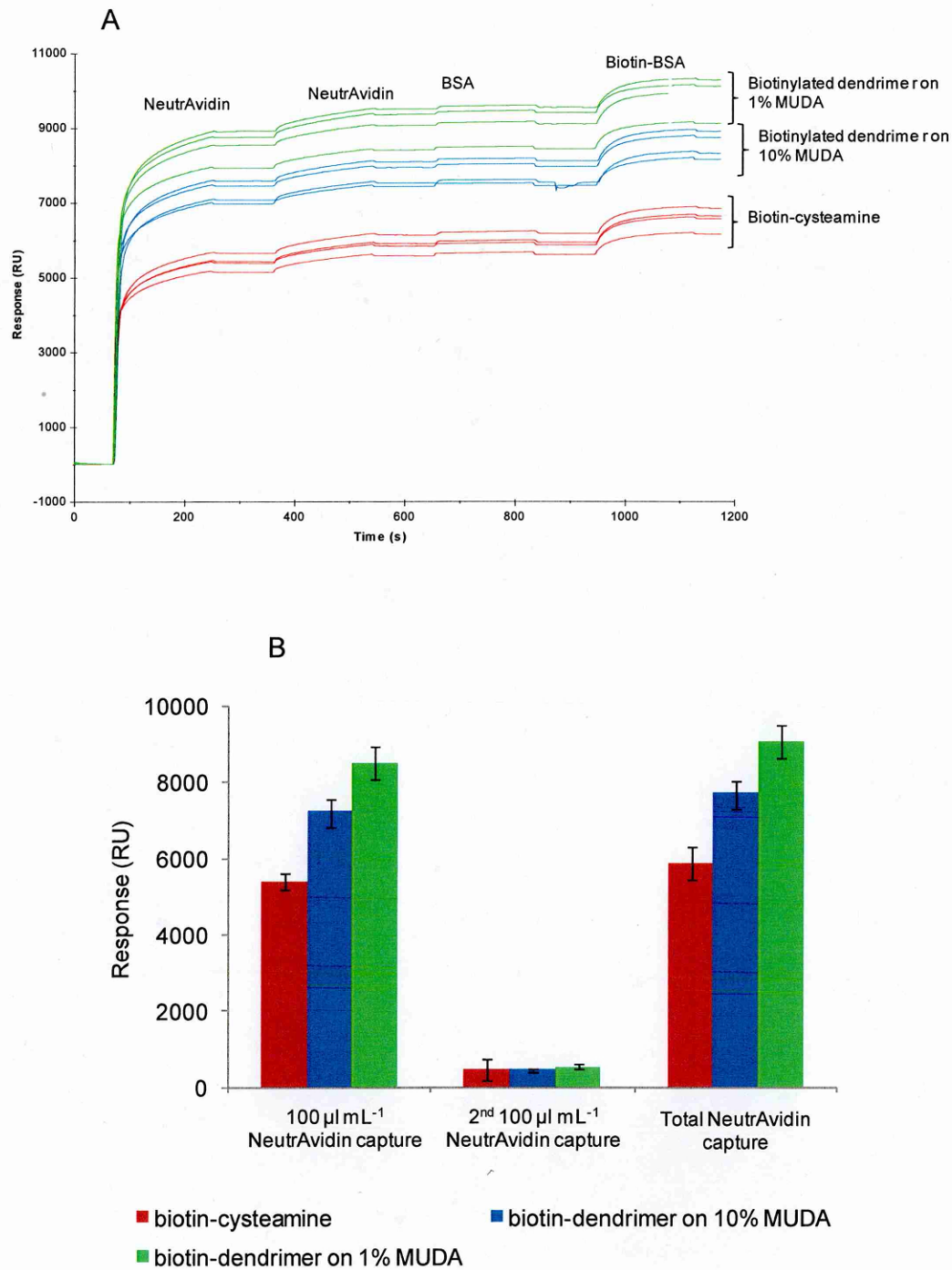


Figure 2.15. Biacore sensor chips were modified with different surfaces and NeutrAvidin capture response obtained from these surfaces were compared.

Table 2.3 The summary of NeutrAvidin immobilisation/capture results using different surface chemistries on Biacore sensor chips.

	NeutrAvidin capture	Ratio
Biotin-cysteamine	5871	-
Biotin-dendrimer on 10% MUDA	7737	$7737/5871 = 1.3$
Biotin-dendrimer on 1% MUDA	9066	$9066/5871 = 1.5$

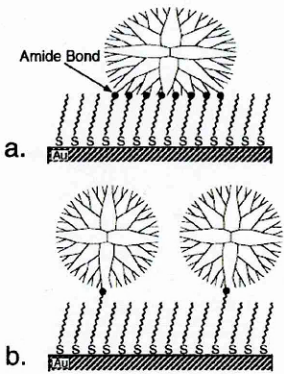


Figure 2.16. Dendrimers immobilised to 100% MUDA surface (a) and dendrimers immobilised to a diluted MUDA surface (b) (Crooks and Ricco, 1998).

In an earlier study in 2004, Mark et al. suggested that dendrimer modified surfaces caused a 2.5 fold antibody immobilisation signal enhancement with respect to a flat SAM surface (Mark et al., 2004). Two recent studies by Singh et al. and Shayir et al. has shown 1.6 times and 2 times higher immobilisation levels using dendrimer coated surfaces, respectively (Singh et al., 2009; Syahir et al., 2009). In our study we observed 1.5 times higher immobilisation levels using dendrimers that was close to the results obtained in recent studies.

After NeutrAvidin capture, binding of $5 \mu\text{g mL}^{-1}$ biotinylated BSA was performed to assess the activity of the captured NeutrAvidin. As it can be seen from Figure 2.15,

BSA non-specific binding resulted in 19 to 38 RU response, indicating that non-specific binding of BSA to the surfaces was limited. A $5 \mu\text{g mL}^{-1}$ Biotin-BSA binding was slightly high on NeutrAvidin captured biotin-dendrimer on 10% MUDA surface (758 ± 43 RU) which was followed with biotin-dendrimer on 1% MUDA surface (701 ± 33) (Figure 2.17). This result indicates that although biotin-dendrimer on 1% MUDA surface had the highest NeutrAvidin capture capacity, biotin-dendrimer on 10% MUDA had higher protein capture capacity. Additionally the non-specific BSA binding on biotin-dendrimer on 10% MUDA surface was lower with respect to other surfaces, therefore this surface might be preferred to immobilise biotinylated proteins.

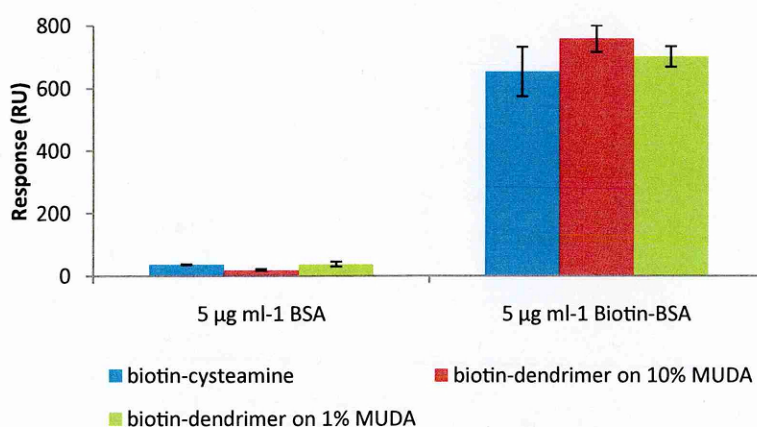


Figure 2.17. Binding of biotin-BSA on NeutrAvidin that were capture on different biotinylated surfaces.

2.4.2.2 Testing of PAMAM amino dendrimer surface for QCMA-1 sensor chips

The capacity of biotinylated dendrimers was higher than the other flat surfaces when employed as SPR sensor surface. The same sensor surfaces were tested using QCMA-1 instrument to investigate the effect of detection method to the performance

of the dendrimer coated sensor surface. Initially a test was devised to see the performance of MUDA and biotinylated-dendrimer surfaces side-by-side using the two sensing spots of a sensor chip. Sensing spot one had a dendrimer surface that was modified with biotin (prepared using method 1, Section 2.3.2), sensing spot two still had 10% MUDA surface. A $100\ \mu\text{g mL}^{-1}$ NeutrAvidin was captured on biotinylated dendrimer layer (sensing spot 1) and immobilised on to the 10% MUDA surface (sensing spot 2) (Figure 2.18).

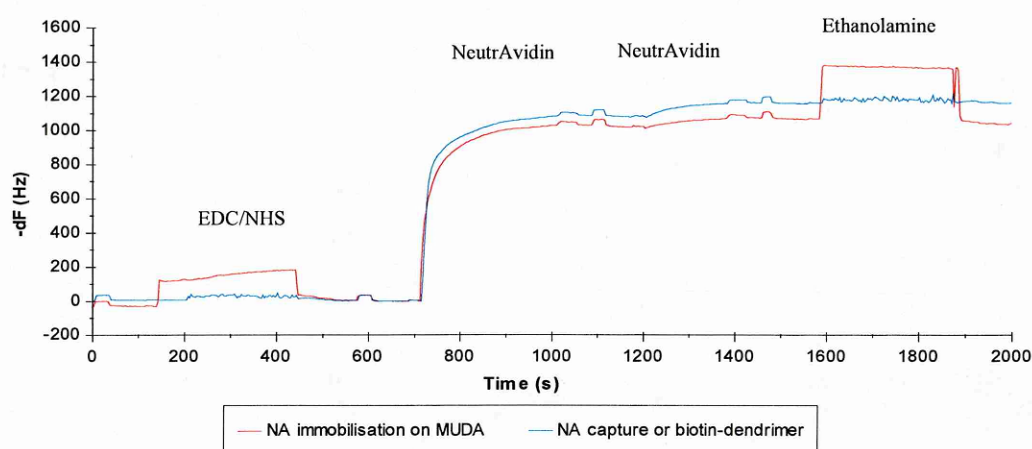


Figure 2.18. Sensing spot 1 of a QCMA-1 sensor chip was modified with biotinylated dendrimer and sensing spot 2 was left as MUDA surface. Later NeutrAvidin was immobilised on MUDA surface (sensing spot 2) and captured on biotinylated dendrimer surface (sensing spot 1).

For comparison, NeutrAvidin was also captured on to a biotin monolayer (method 3 – Section 2.3.2) (Table 2.4). In terms of NeutrAvidin immobilisation/capture only 6% difference was observed between the biotinylated dendrimer, biotin monolayer or MUDA coated sensor surface.

Table 2.4. The summary of NeutrAvidin immobilisation/capture results using different surface chemistries on a 20 MHz quartz crystal sensor chip.

Sensor surface	NeutrAvidin immobilisation/capture
10% MUDA	1060 Hz
Biotin-cysteamine	1076 Hz
Biotin-dendrimer on 10% MUDA	1150 Hz

These results indicated that for QCM detection, dendrimer coated sensor surfaces used in this format did not increase the surface capacity and hence no benefit of using dendrimers in this format for QCMA-1 biosensors.

2.4.3 Development of UVlink polymer surface for biomolecule immobilisation

A polymeric structure was grafted on the substrate surface to provide a new sensor surface prior to biomolecule immobilisation and this new surface was called UVlink. To graft the UVlink polymer layer on solid matrices, free radical chain polymerisation by means of a UV light was utilised. The UVlink polymer surface was tested on both SPR and QCM sensors. A patent application was filed for the work in July 2009 (Y. Uludag and I. E. Tothill, High capacity polymer surface for immobilisation of biomolecules; GB0911634.4) and this was re-filed in July 2010.

2.4.3.1 Optimisation of initiator immobilisation

Initiator based polymerisation results in a controlled, ultra-thin grafted polymer layers and does not result in gelation of the polymerisation mixture unless the initiator is used in the solution instead of on the sensor surface. Initially the iniferter activation step was optimized and this was followed by the optimisation of the polymerization process. The initiator activation conditions were optimized by changing the initiator concentration and UV irradiation time. BAC coated sensor chips were placed to Petri dishes containing three different concentrations of initiator (200 mM, 50 mM and 10 mM) and irradiated for 20 minutes with UV light. This was followed with 40 minutes immobilisation with crosslinker and monomer mixture. The thicknesses of the polymers were measured using an ellipsometer and the results were compared (**Figure 2.19**). The average thickness of the polymers was ca. 3 nm, and no significant difference was found between them. Therefore a 10 mM initiator concentration was chosen for further assays.

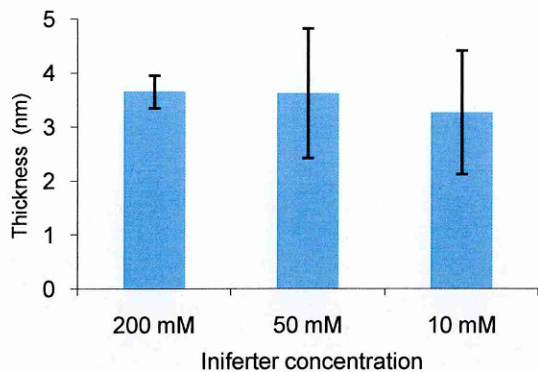


Figure 2.19. The thicknesses for the polymer layers that were fabricated using varying concentrations of the initiator were measured using an ellipsometer (n=2).

To optimise the activation time BAC coated sensor chips were placed to Petri dishes containing 10 mM initiator solution and irradiated for 20 minutes and 40 minutes with UV light. This was followed with 40 minutes immobilisation with crosslinker and monomer mixture. The thickness of the sensor chips were again ca. 3nm for both 20 minutes and 40 minutes activated sensor chips (Figure 2.20). This result indicated that 20 minutes of activation with initiator was sufficient.

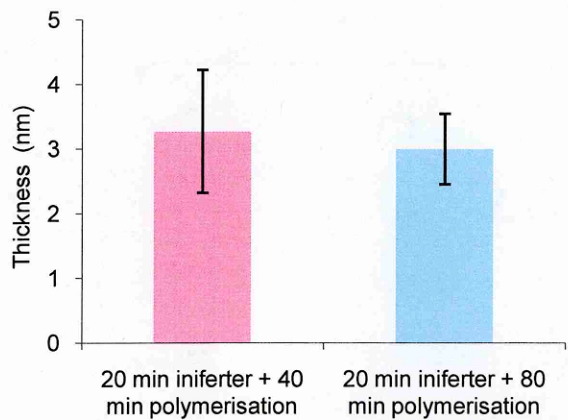


Figure 2.20. The polymer thickness was measured using ellipsometer after the use of different activation time with the initiator (n=2).

After the optimisation of initiator activation step the polymerisation time was increased from 40 minutes to 80 minutes to obtain thicker polymer layer and to investigate if it was possible to control the thickness by changing the polymerisation time. The thicknesses obtained from 40 minutes polymerisation and 80 minutes polymerisations were not different from each other (ca. 3 nm; Figure 2.20). This result indicated that the polymerisation did not continue after 40 minutes possibly due to inactivation of the initiator. To investigate this, after 40 minutes of polymerisation the sensor chips were again activated with the initiator solution and a further 40 minutes of polymerisation was performed. This time the thickness of the sensor chips reached 7.2 nm (Figure 2.21).

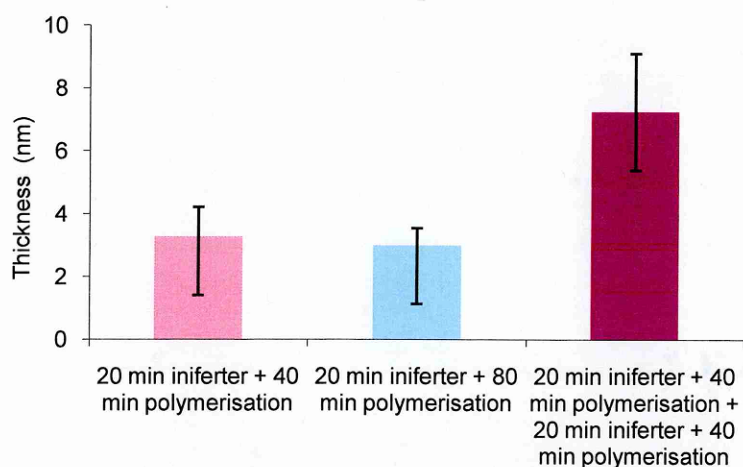


Figure 2.21. The polymer thickness results after 40 minutes and 80 minutes of polymerisation.

These results clearly show that the iniferter was no longer active after 40 minutes of polymerisation. The possible cause of this in-activation could be the oxygen, since the experiments were performed in open air environment. To perform the polymerisation in an oxygen-free environment, the UV device was placed in an

Atmosbag and it was continuously flushed with argon. After 20 minutes activation with 10 mM initiator, the polymerisation was performed for 40 minutes, 60 minutes and 80 minutes. Surprisingly 40 minutes of polymerisation did not result in thicker than 1 nm layer; however, 60 minutes of polymerisation yielded 16 ± 1 nm and 80 minutes of polymerisation produced 32 ± 2 nm thick polymer layers (Figure 2.22). The difference of the thickness for 40 minutes of polymerisation in air and in Atmosbag could be the effect of temperature on the polymerisation process. Higher temperature increases the speed of polymerisation, since the Atmosbag was continuously flushed with argon the environment was cooler than the experiment in open air. This could also have contributed to the change in polymerisation efficiency. To be able to control the polymerisation and obtain reproducible results, a temperature control environment is recommended. In conclusion a draft protocol was obtained for the fabrication of ultra-thin polymer layers using this procedure.

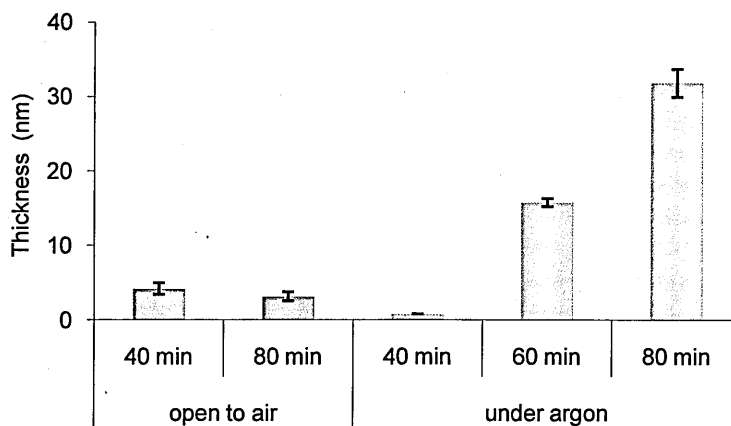


Figure 2.22. The polymer thickness versus time plot for photo-polymerised layers.

A further optimisation of the polymerisation variables, such as, crosslinker, monomer concentrations and ratios were carried out.

2.4.3.2 UVlink polymer for surface plasmon resonance sensor chips

Bare gold Biacore sensor chips were coated with UVlink polymer surface using a polymerization mixture containing 1 mL (5%) methacrylic acid (monomer), 0.1 mL (0.5%) ethylene glycol dimethacrylate (EDMA; crosslinker), 2 mL (10%) deionised water and 16.9 mL toluene. AFM images of the UVlink coated sensor chip was obtained using Dimension 3000 SPM instrument in tapping mode and a silicon probe (type: PPP-NCH-50) was employed for imaging. The roughness of the polymer is calculated as 13.4 nm (Rms) and depth of the polymer according to AFM image is around 100 nm (Figure 2.23 - A). The phase angle plot of the AFM image indicates that the polymer surface obtained is uniform in surface properties (Figure 2.23 - B).

Functional assay using the surface grafted sensor chips were performed using a Biacore 3000 instrument according to the protocol described in Section 2.3.8. Rabbit anti-mouse IgG immobilised sensors produced an average response change of 5540 ± 283 RU and mouse IgG immobilised sensors produced a response change of 4805 ± 49 RU (Figure 2.20). After the immobilisation step the functionality of the sensors was tested by injection $5 \mu\text{g mL}^{-1}$ mouse IgG to obtain a binding signal from the rabbit anti-mouse IgG immobilised sensor surfaces and to obtain a background signal from the mouse IgG immobilised sensor surfaces; 513 ± 18 RU and 14 ± 3 RU obtained respectively from the results (Figure 2.25).

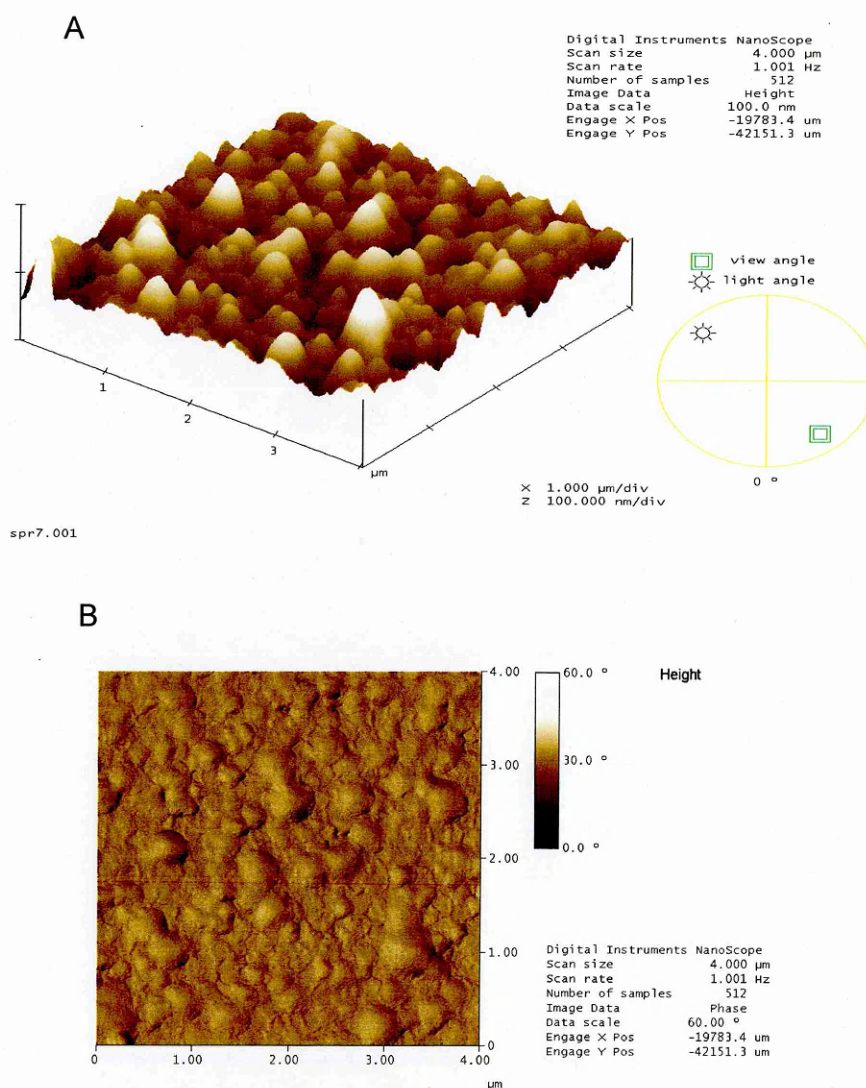


Figure 2.23. (A) AFM image of a polymer coated Biacore 3000 sensor chip. (B) Phase shift AFM image obtained with tapping mode of imaging the polymer coated Biacore 3000 sensor chip.

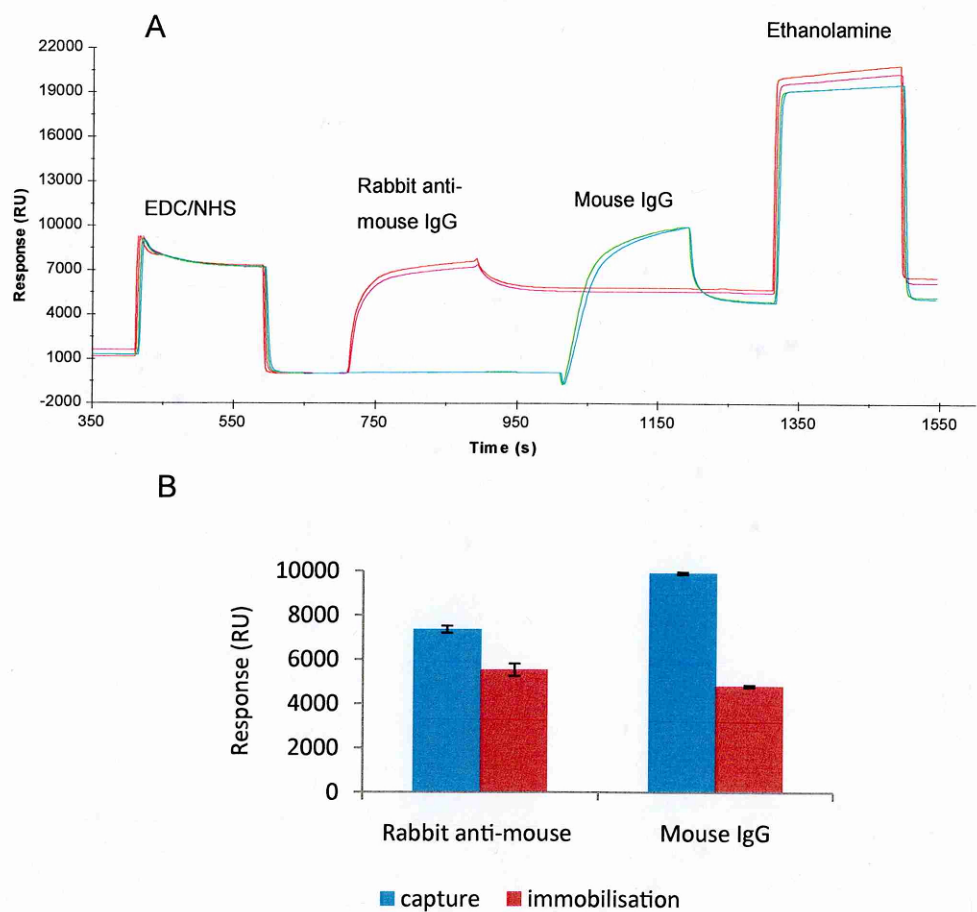


Figure 2.24. Immobilisation of rabbit anti-mouse and mouse IgG antibodies on to UVlink polymer coated SPR sensor chip.

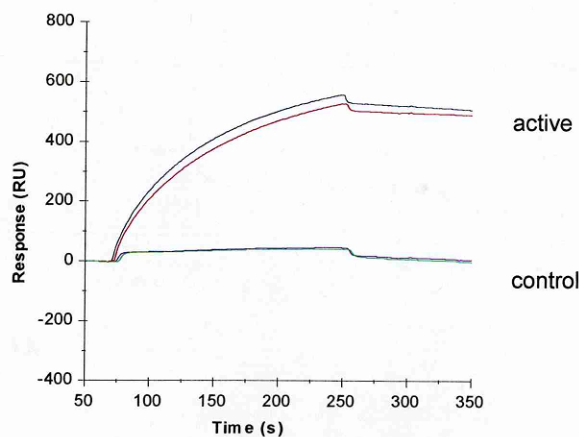


Figure 2.25. Binding of mouse IgG on to rabbit anti-mouse IgG immobilised active surfaces and mouse IgG immobilised control surfaces using a Biacore 3000 biosensor sensor chip coated with the UVlink polymer.

The polymer fabrication protocol was then repeated to improve the process and improve the reproducibility. For the initial tests, the polymer surfaces fabricated on the SPR sensor chips were visible to the naked eye but the surfaces were coated with a non-uniform polymer layer; the polymerization conditions for these experiments are given in Table 2.5. As the Biacore SPR chips have a larger surface area than QCMA-1 sensor chips the non-uniformity of the layer was more apparent. A standard biological assay was performed using these polymer coated SPR sensor chips, however low antibody immobilization (down to ~430 RU) and antigen binding (down to ~100 RU) responses were obtained with respect to the earlier results as shown in Figure 2.26.

Table 2.5. The experimental conditions of the UVlink polymers fabricated using methacrylic acid as monomer and EDMA as crosslinker.

	Polymer A	Polymer B	Polymer C	Polymer D	Polymer E
Polymerisation mixture	5 % monomer + 0.5 % crosslinker + 10 % deionised water	5 % monomer + 0.5 % crosslinker + 10 % deionised water	5 % monomer + 0.5 % crosslinker + 10 % deionised water	5 % monomer + 1 % crosslinker + 10 % deionised water	5 % monomer + 1 % crosslinker + 10 % deionised water
Starting temperature	not known	not known	not known	21 °C	22 °C
Polymerisation time	60 min	60 min	60 min	60 min	90 min

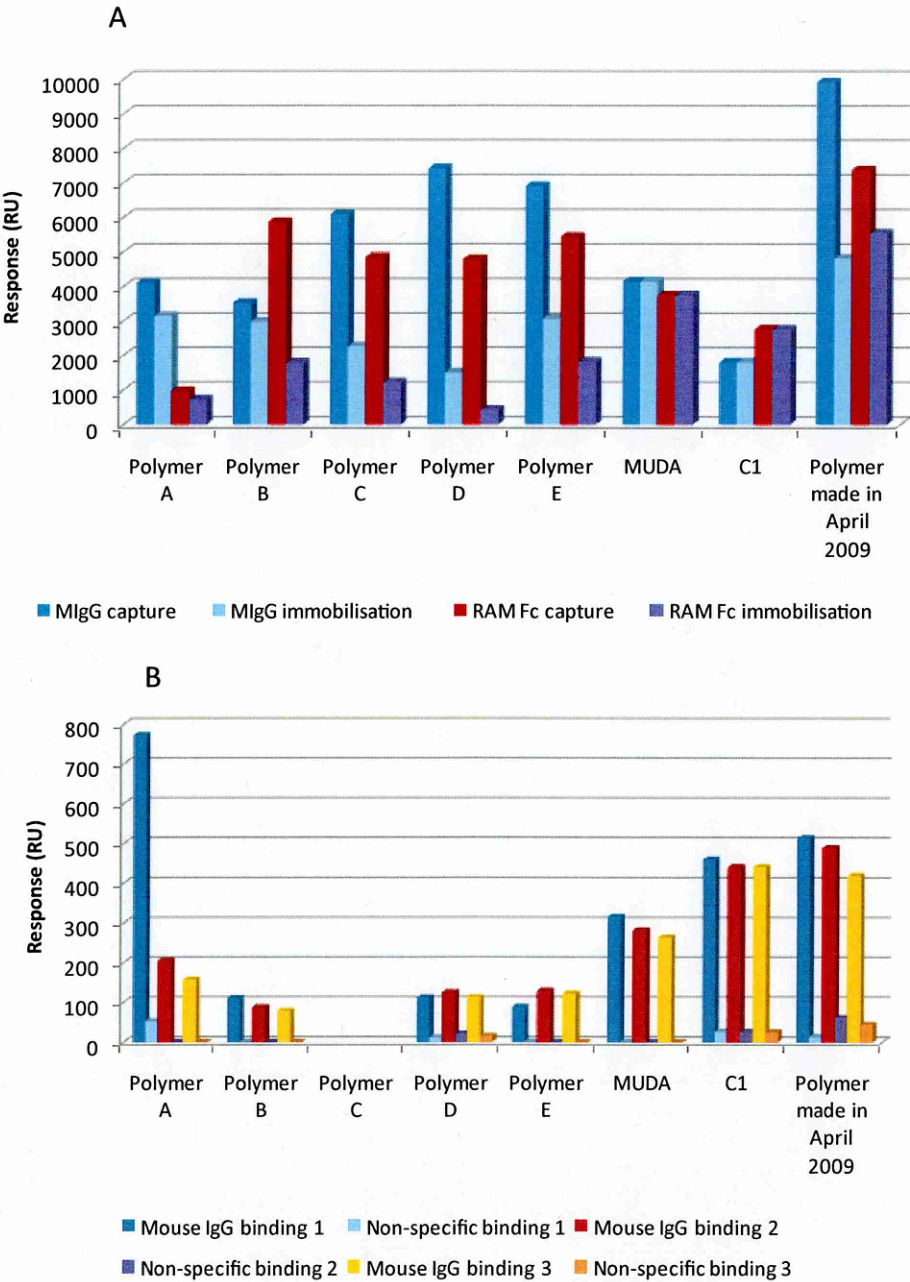


Figure 2.26. (A) Mouse IgG and Rabbit anti-mouse IgG were immobilised on to UVlink polymer coated SPR sensor chips. (B) Later $5 \mu\text{g mL}^{-1}$ mouse IgG were injected on to flow cells for three minutes and the binding was measured. (C1-Biacore 3000 sensor chip)

At this stage the possible reasons for irreproducible and non-uniform UVlink polymer surfaces were investigated. Two factors were found to contribute to polymerization efficiency to a great extent and these are polymerization temperature and the sonication of water-toluene mixture. Initially the temperature of the Atmosbag was recorded from the beginning to the end of polymerization process. Lab temperature was measured as 21-22 °C so initiator immobilization and the first parts of the polymerization started at this temperature (Figure 2.27). This low starting temperature may cause a much slower polymerization till the temperature reaches 25 °C or more. Day to day and seasonal temperature fluctuations does influence the polymerization performance and causes irreproducibility of the results obtained. As at this time we are unable to conduct this experiment in a temperature controlled room due to the toxicity of the chemicals used and therefore we have to use a flow cabinet, so it was decided to start the polymerization after the temperature of Atmosbag reaches 25 °C. In winter months, this simply can be achieved by switching on the UV device for a while prior to polymerization.

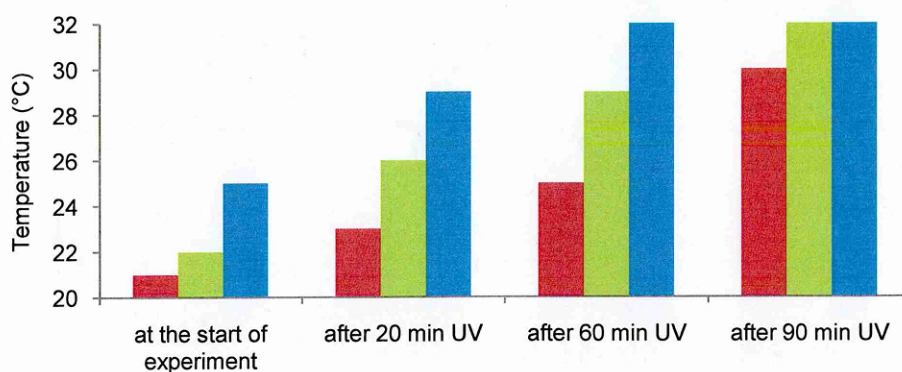


Figure 2.27. At the start of the experiment the temperature in the Atmosbag reflected the temperature of the environment for the first two experiments (shown as red and green columns). For the third experiment the UV device was switched on prior to polymerization to increase the temperature in the Atmosbag to 25 °C, then the polymerization was started (blue column).

Water and toluene are not miscible to each other as density of water is higher than toluene ($d_{\text{toluene}} = 0.87 \text{ g mL}^{-1}$), it stays below the toluene and a biphasic solution is created. The sonication and nitrogen purge cause water molecules to disperse in toluene to a degree. It is believed that as the monomer and crosslinker are not dissolved in water, these tiny water droplets create small pockets where polymerization does not occur, hence pores within the polymer are formed. Of course the concentration and ratios of both monomer and crosslinker have an additional effect on the polymer structure and the size of the pores within the polymer. By changing the absorbed water quantity in the toluene, it is possible to create polymers with different surface properties. For example 30 s sonication of toluene-water mix results in a cloudy mixture that contains tiny water droplets within the toluene. If this solution directly used for polymerisation, this droplets get bigger by the time and cover the sensor surface (Figure 2.28 - A). This results in a uniformly patterned UVlink polymer surface [Table 2.7, Polymer 3] (Figure 2.28 - B).

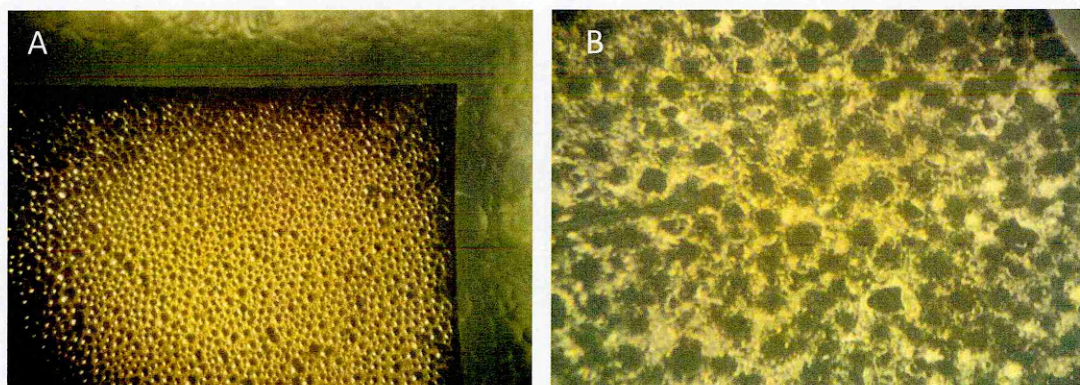


Figure 2.28. (A) Water droplets formed on an SPR sensor chip during polymerisation. (B) This results in a uniformly patterned polymer surface [Table 2.7, Polymer 3]. Images are taken using a microscope (Microoptical Co.; image A: 25 times magnification; image B: 50 times magnification).

Visible and uniform UVlink polymer surfaces were fabricated on SPR sensor chips having different surface properties (flat or uniformly patterned surface). The experimental conditions of the selected polymers can be found from Table 2.7. The polymer coated surfaces were tested with standard antibody immobilisation and antigen binding and the results of these assays can be found from Figure 2.29, Figure 2.30 and Figure 2.31. The fabricated polymer surfaces have similar antibody immobilising but higher antigen binding capacity with respect to MUDA coated SPR chips; higher immobilisation capacity but slightly lower antigen binding capacity with respect to a Biacore C1 sensor chip. Using one of the polymers developed (Polymer 5; rabbit anti-mouse IgG immobilisation 4500 RU) 20 repeated antigen binding assays (393 ± 17 RU) were performed with minimum non-specific binding (14 ± 3 RU).

Table 2.6. The experimental conditions of the UVlink polymers fabricated using methacrylic acid as monomer and EDMA as crosslinker.

	Polymer 1	Polymer 2	Polymer 3	Polymer 4	Polymer 5
Polymerisation mixture	5 % monomer + 0.5 % crosslinker + 10 % deionised water	5 % monomer + 1 % crosslinker + 5 % deionised water	5 % monomer + 1 % crosslinker + 10 % deionised water	5 % monomer + 1 % crosslinker + 10 % deionised water	5 % monomer + 1 % crosslinker + 10 % deionised water
Polymerisation mixture pre-treatment	15 min sonication, 10 min nitrogen stream, 30 s sonication	15 min sonication, 10 min nitrogen stream, 30 s sonication	15 min sonication, 10 min nitrogen stream, 30 s sonication	15 min sonication, 10 min nitrogen stream, 30 s sonication	15 min sonication, 10 min nitrogen stream
Polymerisation time	90 min	70 min	60 min	40 min	90 min
Properties of the polymer formed	Visible, uniform patterned surface	Visible, uniform patterned surface	Visible, uniform patterned surface	Not visible	Visible, uniform flat surface

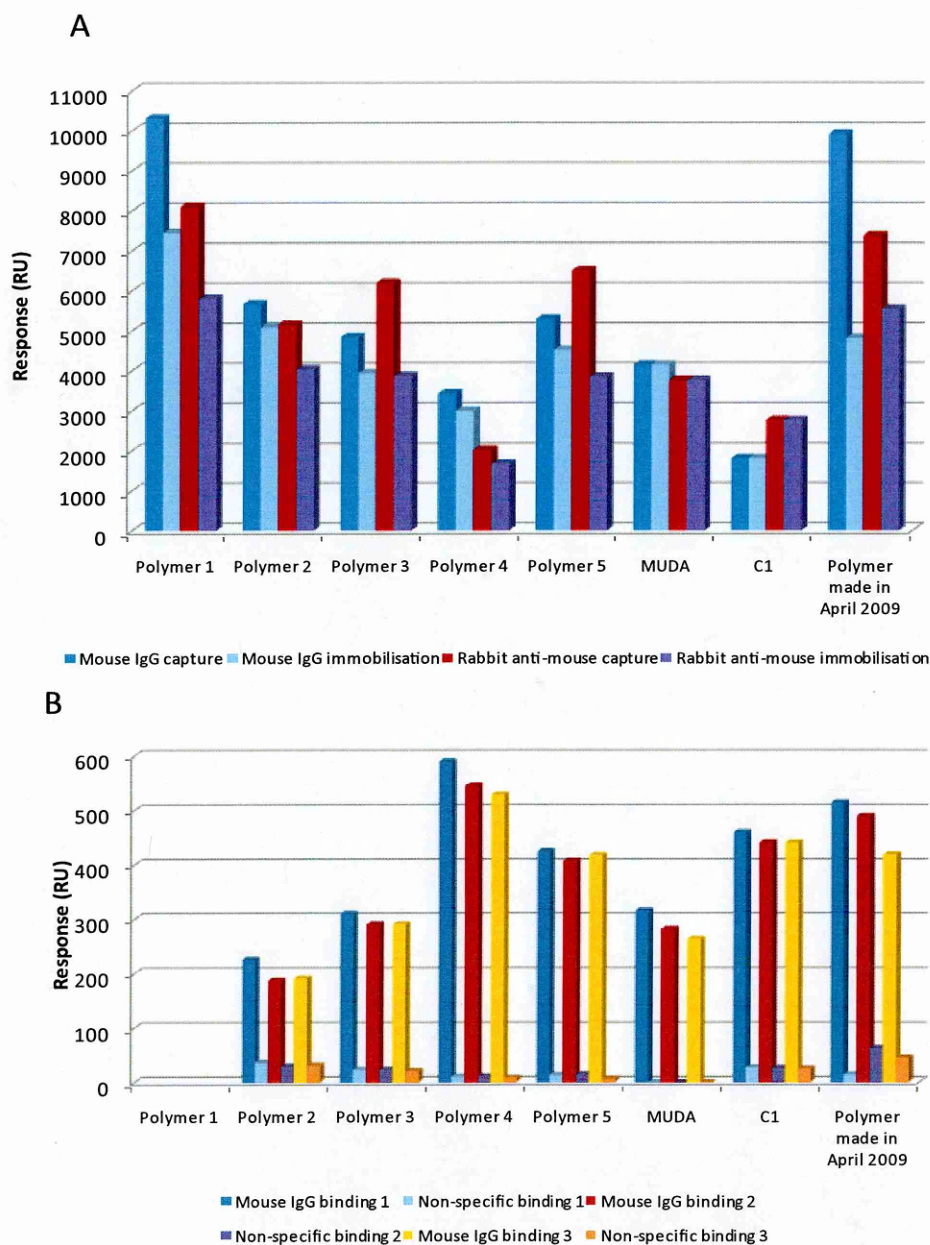


Figure 2.29. (A) Mouse IgG and Rabbit anti-mouse IgG were immobilised on to UVlink polymer coated SPR sensor chips. (B) Later $5 \mu\text{g ml}^{-1}$ mouse IgG were injected on to flow cells for three minutes and the binding was measured. (C1-Biacore 3000 sensor chip) The results of the polymer made in April 2009 was compared to the other polymers fabricated at a later date.

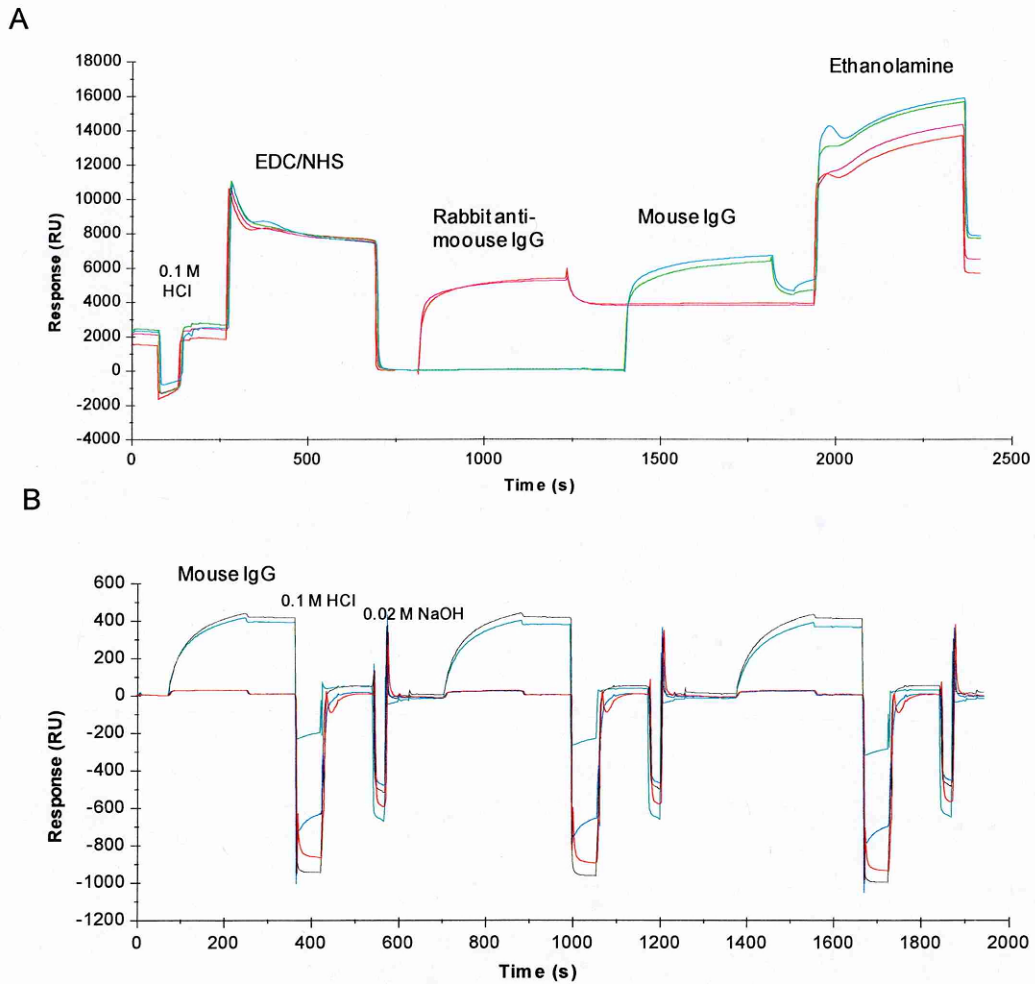


Figure 2.30. (A) Mouse IgG and Rabbit anti-mouse IgG were immobilised on to Polymer 5 coated SPR sensor chip. (B) Later 5 $\mu\text{g mL}^{-1}$ mouse IgG were injected on to flow cells for three minutes and the binding was measured. The green and grey lines of the plot indicate mouse IgG binding on rabbit anti-mouse IgG immobilised flow cells; blue and red lines indicate non-specific binding to mouse IgG immobilised flow cells.

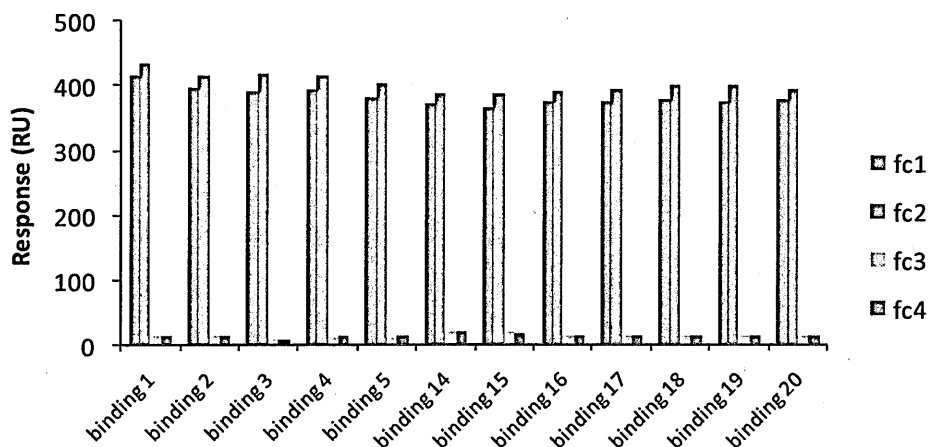


Figure 2.31. The blue (flow channel-fc 1) and red (fc2) columns of the graph indicate the measured response of Biacore 3000 instrument for mouse IgG binding on rabbit anti-mouse IgG immobilised Polymer 5 surfaces; green (fc3) and purple (fc4) columns indicate non-specific binding to mouse IgG immobilised surfaces.

The highest antibody immobilisation does not result in high binding of the antigen every time. If the immobilised antibodies are too crowded the binding sites of the antibodies are most likely to be hindered. That could be the reason that even if the immobilisation of antibodies were lower with the C1 sensor chip, the binding levels are higher. To minimise surface crowding either lower concentrations of antibodies need to be used for immobilisation or the number of carboxyl groups on the polymer surface can be reduced by using a second monomer that does not contain carboxyl groups.

2.4.3.3 UVlink polymer for QCMA-1 biosensor chips

In the assays using Biacore instrument, the immobilization capacity of the UVlink polymers created using just the solvent (DMF or toluene) or solvent plus water were compared and found that use of toluene and water together for polymerization have potential to create high capacity polymer surfaces (Figure 2.32). Water and toluene

are not miscible to each other and as density of water is higher than toluene ($d_{\text{toluene}} = 0.87 \text{ g mL}^{-1}$), it stays below the toluene and a biphasic solution is created. The sonication and nitrogen purge cause water molecules to disperse in toluene to a degree. It is believed that as the monomer and crosslinker are not dissolved in water, these tiny water droplets create small pockets where polymerization does not occur, hence pores within the polymer are formed. Of course the concentration and ratios of both monomer and crosslinker have an additional effect on the polymer structure and the size of the pores within the polymer.

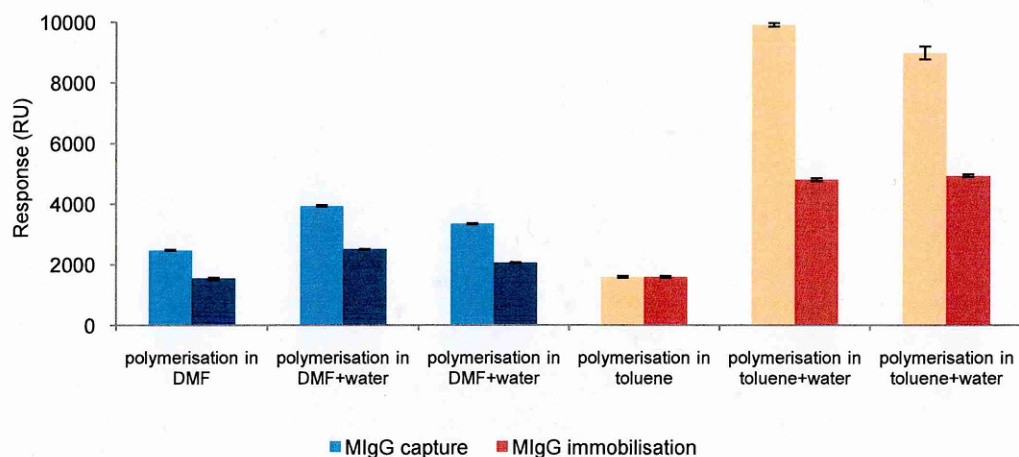


Figure 2.32. The polymers on Biacore sensor chips were fabricated using 0.5% EDMA, 5% methacrylic acid, solvent (DMF or toluene) and 10% water. After polymer formation the sensor chips were docked to the Biacore instrument and mouse IgG immobilization assay was performed. The figure shows the mouse IgG capture and immobilization results using the polymer coated sensor chips.

After these findings from the experiments using Biacore sensor chips, the experiments were carried to the QCM-A-1 platform. The activation of the sensor chip surface using initiator was being performed in DMF, and this solvent was damaging the silver paint that was used to connect the electrodes of the sensor chip. Therefore,

only a small portion of the sensor chips were in working condition after the polymerisation process. Different solvents were tried to replace DMF and finally acetonitrile was found as suitable for the process. The best performing polymer using the Biacore instrument was formed using 5% v/v monomer, 0.5% v/v crosslinker, 10% v/v water and toluene as the solvent. The same polymer was formed on a QCMA-1 sensor chip (chip no 1) and antibody immobilisation assay was performed (Figure 2.33). The addition of EDC/NHS to the sensor surface caused a 1000 Hz of shift to the frequency and 827 ohms of shift to the resistance indicating that a big change occurred to the polymer as carboxyl groups of the polymer were exchanged with the NHS. Rabbit anti-mouse IgG ($100\ \mu\text{g mL}^{-1}$) immobilised sensor surface produced a frequency change of 645 Hz, a resistance change of 157 ohms and mouse IgG ($100\ \mu\text{g mL}^{-1}$) immobilised sensor surface produced a frequency change of 1345 Hz and a resistance change of 239 ohms. After the immobilisation the baseline drift increased as well. If we look at the resistance change due to EDC/NHS activation and antibody immobilisation of a MUDA coated sensor chip, we see that EDC/NHS activation does not cause any resistance change and the resistance change due to antibody immobilisation is not larger than 10 ohms (Figure 2.33). The high resistance changes that occur on the UVlink polymer surface may indicate that the polymer created was not rigid enough and might have changed its conformation (possibly collapsed) during the EDC/NHS activation and antibody immobilisation processes.

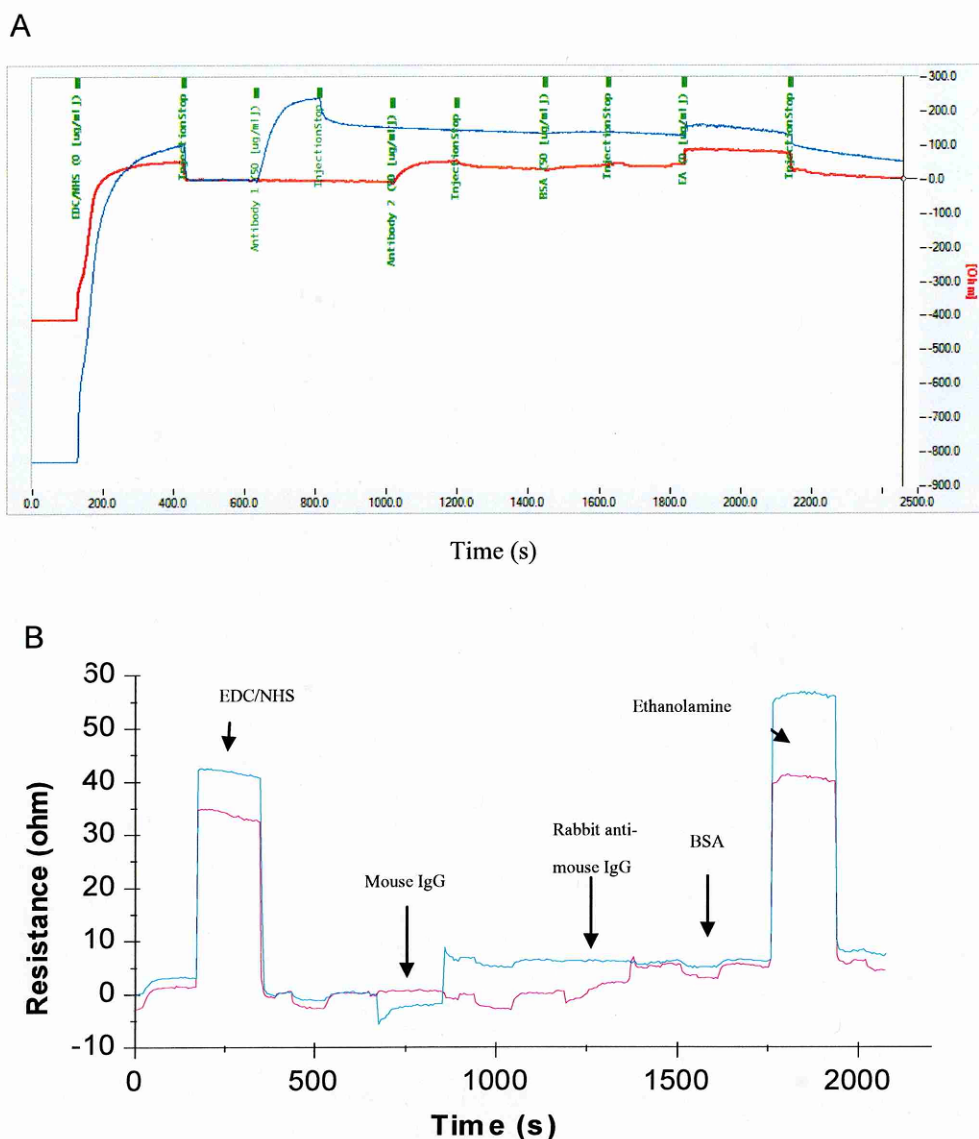


Figure 2.33. Immobilisation responses of rabbit anti-mouse and mouse IgG antibodies in terms of frequency (A) and resistance (B) on the UVlink polymer coated QCMA-1 sensor chip (chip no 1). Polymerisation mixture contains 5% methacrylic acid, 0.5% EDMA, 10% water and toluene as the solvent.

Use of water as an immiscible solvent in the polymerisation mixture helps to create porous polymer on the sensor surface, however due to polymers un-rigid structure its use for the QCMA-1 instrument is problematic and hence the rigidness of the polymer needs to be improved. For that purpose in the next set of experiments after the polymerisation, sensor chips were kept in methanol solution overnight and then

dried overnight at room temperature. The polymers prepared (chip no 3) was tested and found that the resistance change due to EDC/NHS activation antibody immobilisation was much lower than the previous polymers created. This indicates that methanol treatment helps to stiffen the polymer created on the sensor surface. However this time the immobilisation capacity gets lower therefore it is thought that the polymer surface may not be suitable for QCMA-1 sensor chips.

Several UVlink polymer surfaces were fabricated on QCMA-1 sensor chips and used for biomolecule immobilisation and further binding assays. The QCM biosensor is very sensitive to the rigidity of the polymer surface grafted on the sensor surface and this may affect the responses obtained from a binding event. It was very difficult to obtain a rigid surface while increasing the pore size of the UVlink polymer for improving biomolecule immobilisation capacity. Therefore it is concluded that UVlink polymer surface was not yet suitable for QCMA-1 sensor chips and as a future work this can be studied. Some of the assays performed using QCMA-1 platform were summarised in the Table 2.7 and Figure 2.35.

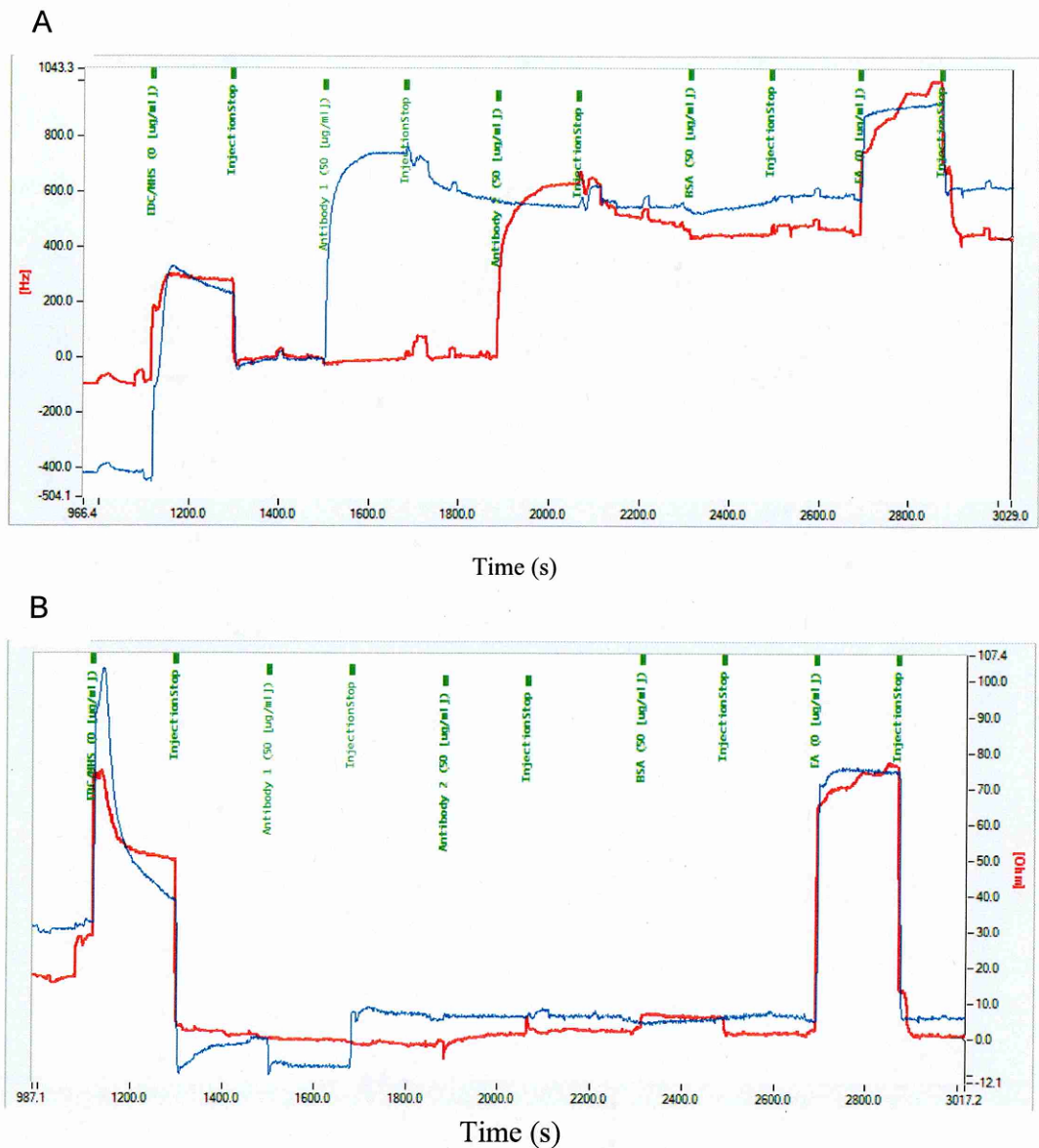


Figure 2.34. Immobilisation responses of rabbit anti-mouse and mouse IgG antibodies in terms of frequency (A) and resistance (B) on the polymer coated QCMA-1 sensor chip (no 3).

Table 2.7. The summary of the polymerisation conditions of the UVlink coated sensor chips and biological assay results. (Monomer: methacrylic acid, crosslinker: EDMA)

Chip number	Chip 1	Chip 2	Chip 3	Chip 4	Chip 5	Chip 6
experiment details	38% Crosslinker & 4% monomer + iniferter rxn in DMF +1 hr rxn in DMF	0.5 % crosslinker & 5 % monomer + iniferter rxn in DMF +1 hr rxn in toluene	0.5 % crosslinker & 5 % monomer + iniferter rxn in acetonitrile +1 hr rxn in toluene	2 % crosslinker & 5 % monomer + iniferter rxn in acetonitrile +1 hr rxn in toluene	1 % crosslinker & 10 % monomer + iniferter rxn in DMF +0.5 hr rxn in toluene + 18 hr methanol treatment	1% crosslinker & 10 % monomer + iniferter rxn in DMF +1 hr rxn in toluene + 18 hr methanol treatment
MlgG capture (Hz)	800	2970	1889	855	-	842
MlgG immobilisation (Hz)	692	1540	1345	749	-	560
RAM Fc capture (Hz)	460	1730	929	605	1563	700
RAM Fc immobilisation (Hz)	432	825	634	500	1478	499
Binding 1 (Hz)	182	100	2	155	226	217
control 1 (Hz)	30	-	-101	-9	-	1
Binding 2 (Hz)	180	-	39	124	220	212
Control 2 (Hz)	60	-	-79	-12	-	42
Binding 3 (Hz)	159	-	124	116	-	186
Control 3 (Hz)	34	-	-37	-13	-	13

Chip number	Chip 1	Chip 2	Chip 3	Chip 4	Chip 5	Chip 6
EDC/NHS profile	normal	1000 Hz freq change	1000 Hz freq change	normal	normal	normal
Drift problem	no	yes	yes	yes	no	no
Resistance-EDC/NHS (ohms)	-	-	827	400	20	26
Resistance-MIgG capture (ohms)	-	-	239	230	-	4
Resistance-MIgG immobilisation (ohms)	-	-	157	180	136	6

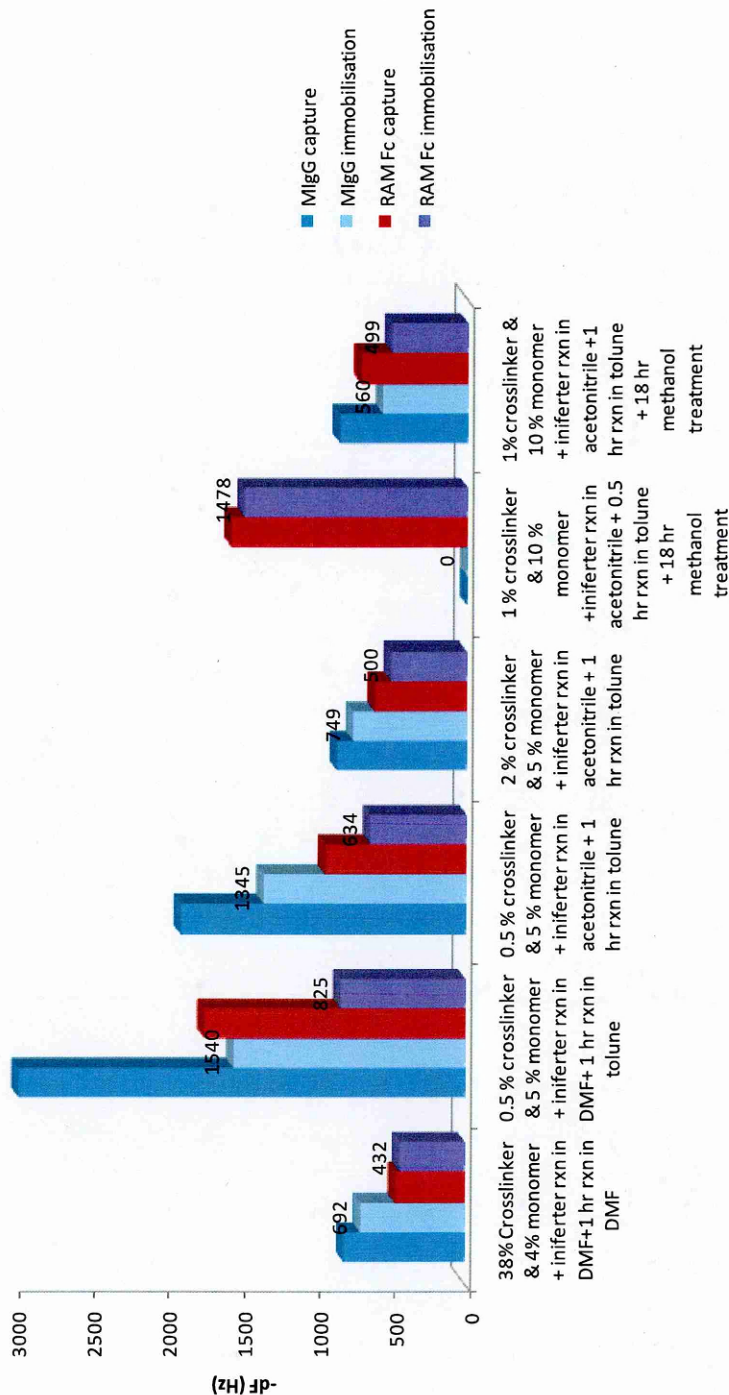


Figure 2.35. The UVlink polymers on Biacore sensor chips were fabricated using crosslinker, monomer, solvent (DMF or toluene) and 10% water. After polymer formation the sensor chips were docked to the QCMA-1 instrument and antibody immobilization assay was performed. The figure shows the mouse IgG and rabbit anti-mouse IgG capture and immobilization results using the polymer coated sensor chip.

2.4.4 Development of carboxymethyl dextran surface

CM5 sensor chip developed by Biacore-GE Healthcare has a very high biomolecule immobilisation capacity with respect to monolayer surfaces and till recently was protected by a patent (US Patent No: 5,242,828). As this patent was recently expired, the use of this surface for SPR-2 instrument sensor chips (Sierra Sensors GmbH) was investigated. CM5 sensor chip consists of a carboxymethyl dextran polymer deposited on the sensor surface and fabrication of this surface using different methods on SPR2 sensor chips was studied.

2.4.4.1 Amide-linked CMD surface

Amide-linked CMD surfaces were fabricated on Biacore 3000 and SPR2 sensor chips by immobilising sodium salt of carboxymethyl dextran (MW 10 000 – 20 000) to the amino-thiol coated sensor surfaces by means of conventional EDC/NHS chemistry. Initially CMD surfaces were fabricated by either in-line in the instrument or static in a Petri Dish and later using a Biacore 3000 instrument a functional assay was performed with rabbit anti-mouse IgG and mouse IgG. CMD surfaces prepared from both methods gave very similar antibody immobilisation and binding responses as seen from Figure 2.36. As a next step new sensor chips were fabricated using static coating method and a bromoacetic acid activation step was added to increase the number of carboxyl groups of the dextran surface. This time as expected, higher antibody immobilisation results were obtained from both Biacore 3000 and SPR2 sensor chips. Although the mouse IgG binding on rabbit-anti-mouse IgG responses of Biacore sensor chips were reasonably high, the same higher responses was not obtained from the SPR2 sensor chips.

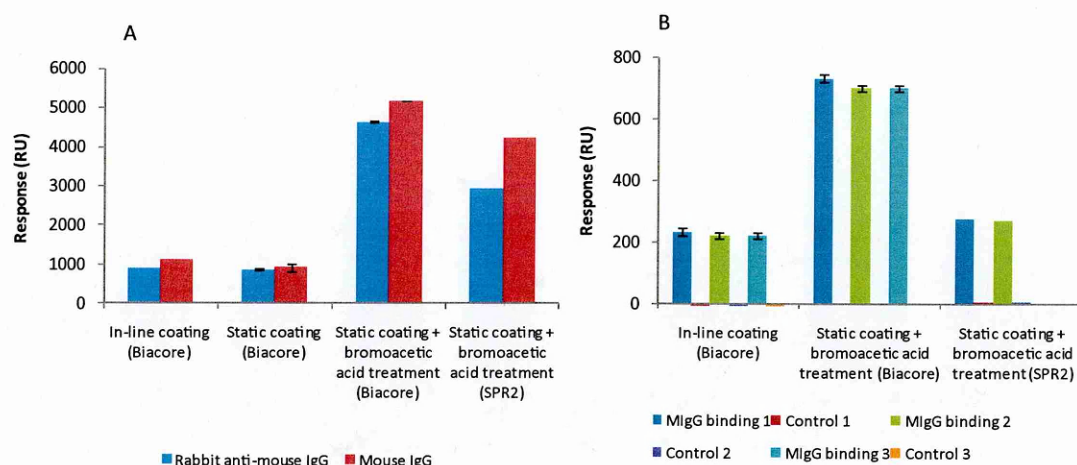


Figure 2.36. (A) Immobilisation of rabbit anti-mouse IgG and mouse IgG on amide-dextran coated Biacore and SPR2 sensor chips. Amide-dextran (MW 10,000 – 20,000) was immobilised on amino groups of the sensor surface using EDC/NHS chemistry either in-line in the instrument or in static conditions in a Petri Dish. (B) 5 $\mu\text{g mL}^{-1}$ Mouse IgG binding on rabbit anti-mouse IgG (active) and mouse IgG (control) immobilised surfaces.

These results clearly show that it is possible to obtain a CMD coated sensor chip using amide-dextran method. The bromoacetic acid step can be avoided if CMD used has high number of carboxyl groups. There are protocols that describe the preparation of CMD from dextran that can be used to prepare CMD with required molecular weight unless a suitable CMD is found commercially. Amide-dextran method is simple, short, does not require many chemicals and contains fewer steps with respect to epoxy-dextran method.

2.4.4.2 Epoxy-linked CMD surface

Epoxy-linked dextran surfaces were prepared on Biacore 3000 and SPR2 sensor chips according to the protocol described at section 2.4.4.2 (bromoacetic acid step method 1). A functional assay with rabbit anti-mouse IgG and mouse IgG was

performed on dextran coated sensor chips and the results were compared with Biacore CM5 sensor chips. For the first batch of epoxy-dextran coated Biacore sensor chips, it was found that while the electrostatic capture of antibodies were higher for the Biacore CM5 chip, the immobilisation of antibodies produced similar responses as seen from the Figure 2.37 - A and Figure 2.38 - A. However, mouse IgG binding on rabbit anti-mouse IgG immobilised epoxy dextran surface is ca. 29 % lower with respect to the rabbit anti-mouse IgG immobilised CM5 sensor chip (Figure 2.37 - B and Figure 2.38 - B). Some of this lower response can be explained by 10 % lower immobilisation of rabbit anti-mouse IgG on the epoxy dextran surface. As the electrostatic capture of the antibodies on epoxy-dextran surfaces were also lower with respect to the CM5 sensor chip, it was thought that the number of carboxyl groups on the epoxy-dextran surface was lower and dextran did not swell in buffer as much. Hence epoxy-dextran surface had lower porosity and lower binding capacity. To increase the number of carboxyl groups, bromoacetic acid step of the fabrication need to be optimised further.

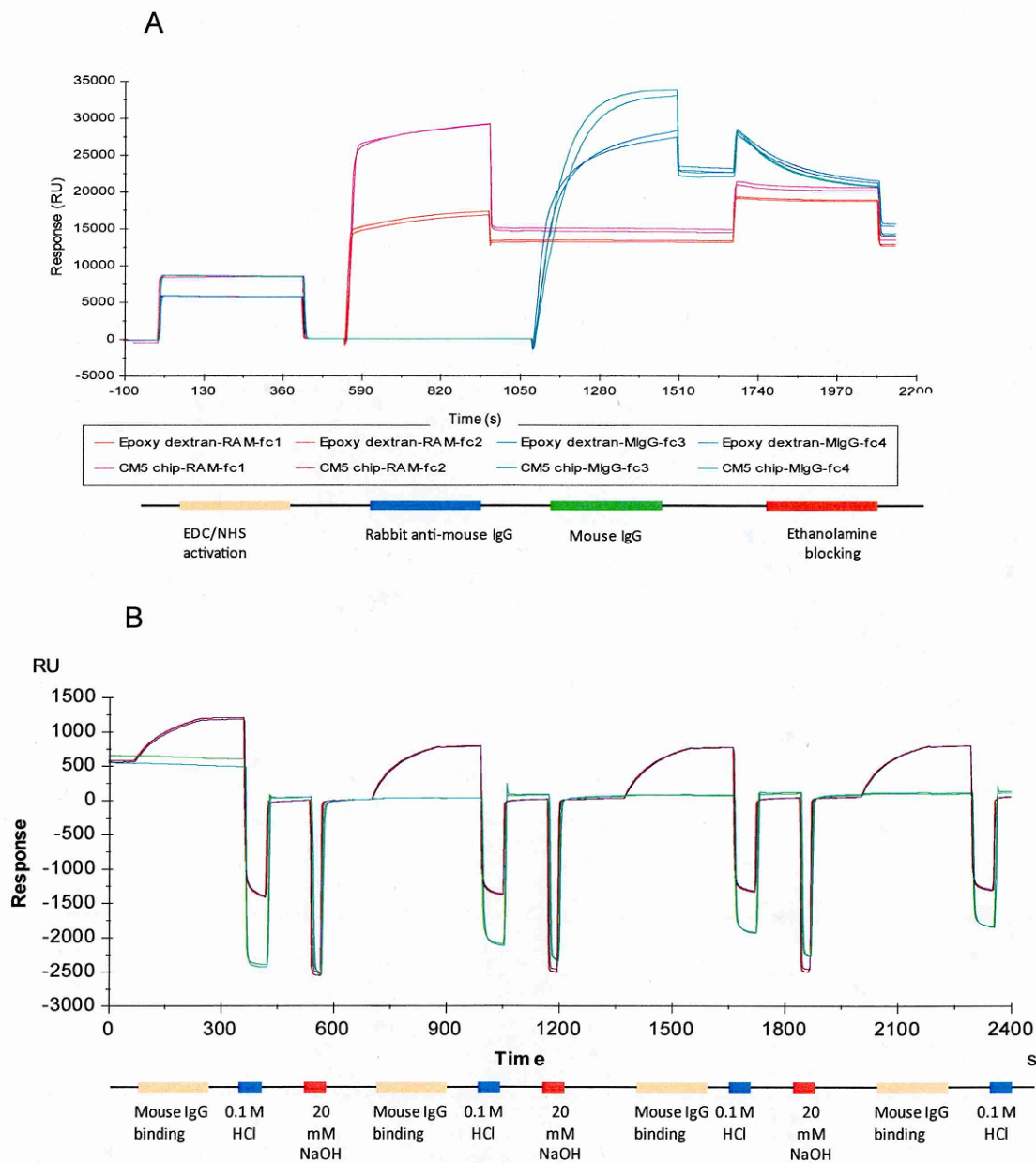


Figure 2.37. (A) Immobilisation of rabbit anti-mouse IgG (RAM Fc) and mouse IgG on epoxy-dextran and CM5 sensor surfaces. (B) Mouse IgG binding on rabbit anti-mouse IgG (active) and mouse IgG (control) immobilised surfaces.

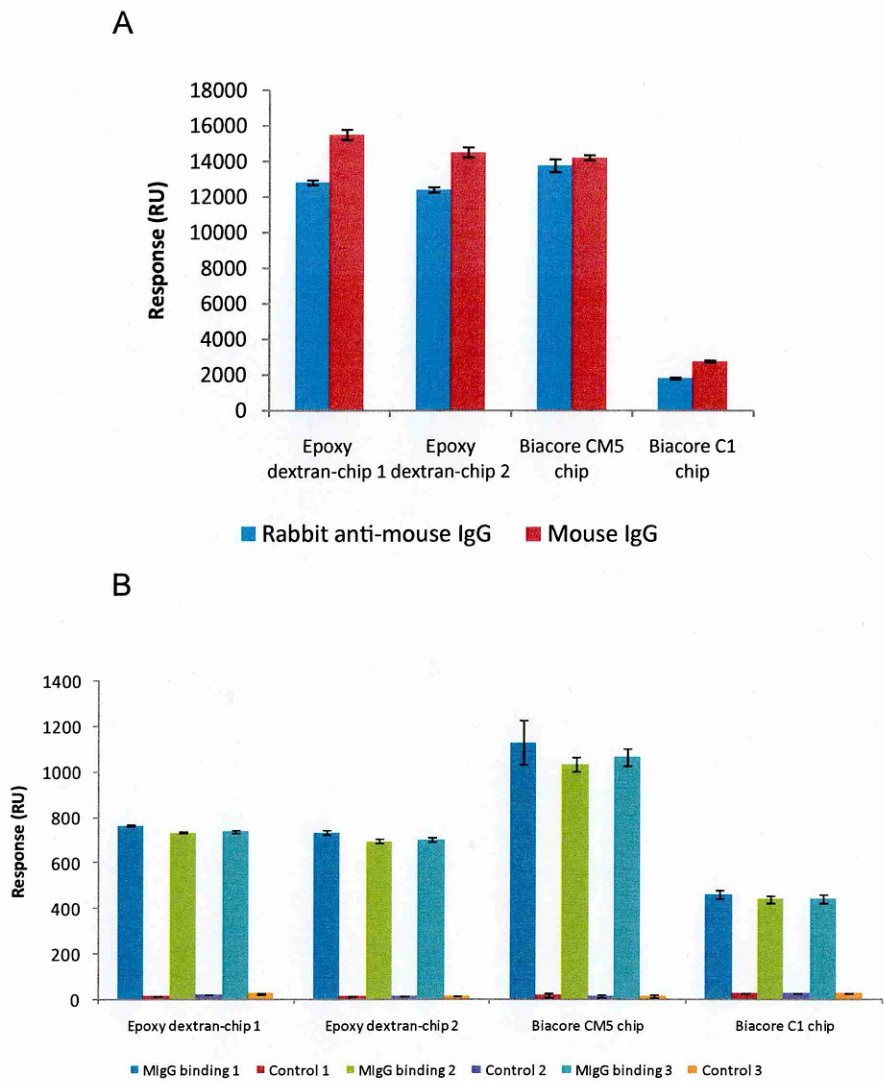


Figure 2.38.(A) Immobilisation of rabbit anti-mouse IgG (RAM Fc) and mouse IgG on dextran surfaces. (B) Mouse IgG binding on rabbit anti-mouse IgG (active) and mouse IgG (control) immobilised surfaces.

As another batch, SPR2 sensor chips and for comparison Biacore 3000 sensor chips were coated with CMD by epoxy-dextran method. From the results of the previous batch it was thought that bromoacetic acid step needs optimisation to increase the level of carboxylation. Therefore, this time a longer protocol was applied for bromoacetic acid step of the fabrication (Section 2.4.4.2, bromoacetic acid step method 2). However, both the immobilisation and binding responses of the Biacore

sensor chips were lower with respect to the results obtained from the first set of experiments described above additionally the responses of SPR2 sensor chips were lower with comparison to Biacore sensor chips (Figure 2.39).

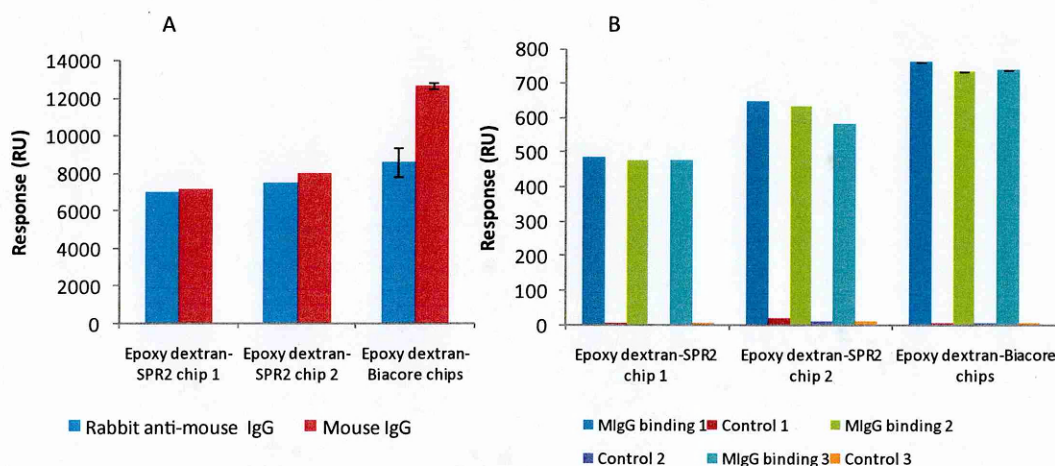


Figure 2.39. (A) Immobilisation of rabbit anti-mouse IgG and mouse IgG on epoxy-dextran coated Biacore and SPR2 sensor chips. (B) 5 $\mu\text{g mL}^{-1}$ Mouse IgG binding on rabbit anti-mouse IgG (active) and mouse IgG (control) immobilised surfaces.

From these results it is clear that especially the bromoacetic acid step of the fabrication needs optimisation to enhance the capacity of the dextran surface.

2.4.4.3 Disturbances to the flow while using dextran chips with SPR-2 instrument

SPR2 instrument has a camera that enables the visualisation of the flow cell during the assay. It was observed that 5-10 minutes after the buffer flow started, both amide-linked and epoxy-linked dextran surfaces started to develop some particles in the flow cell (Figure 2.40). These particles were presumed to be loose dextran that started to swell in buffer flow and move in the flow cell causing disturbances to the flow which could be observed as trace disturbances while performing an assay. To prevent these loose dextran particles, the washing step after dextran immobilisation

needs to be improved. One way to prevent this problem is to leave the sensor chips in deionised water for 15-20 minutes to allow swelling of the dextran which may be followed by vigorous washes with deionised water at 50 °C. It should be also noted that the Biacore sensor chips prepared using the previously described methods (both amide-linked or epoxy-linked dextran) did not caused any disturbances on the assay trace.

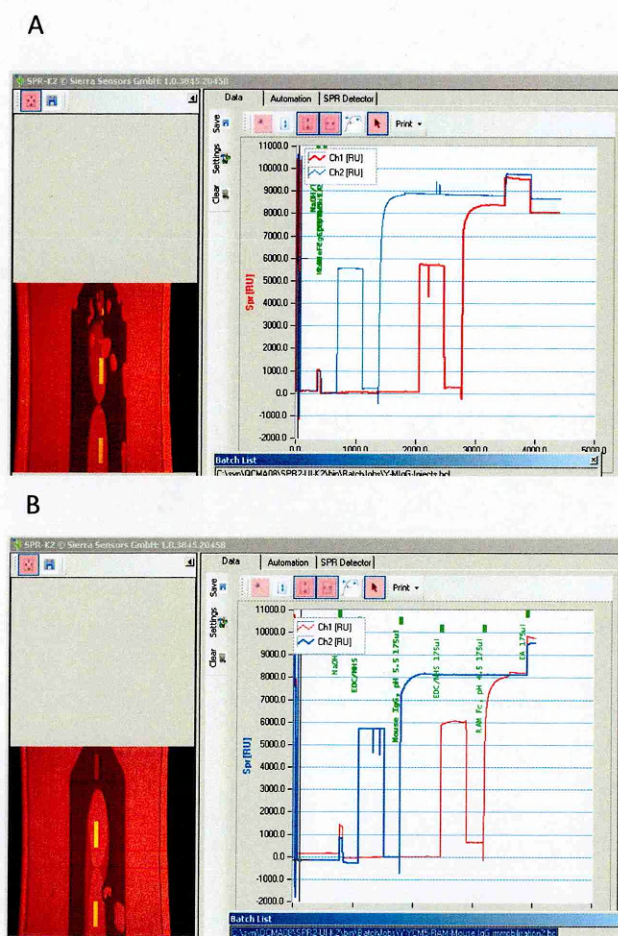


Figure 2.40. The formation of free moving dextran particles while performing assays on two SPR2 sensor chips (A and B).

The problem with the SPR sensor chips may be the result of larger surface area of the flow cell with respect to Biacore flow cell. This problem might have also contributed to the relative lower functional assay responses on the SPR2 sensor chips. It is expected to obtain better results from the dextran coated SPR2 sensor chips after the washing steps of the dextran coating procedure was improved.

2.4.4.4 Amide-linked CMD surface for QCMA-1 sensor chips

Amide-linked CMD surfaces were fabricated on QCMA-1 sensor chips by immobilising sodium salt of carboxymethyl dextran (MW 10 000 – 20 000) to the amino-thiol coated sensor surfaces by means of conventional EDC/NHS chemistry and later conversion of sodium salt of dextran to carboxyl groups by means of a bromoacetic acid activation step. As seen from Figure 2.41, the two sensor chips coated with amide dextran resulted in different antibody immobilisation and binding responses.

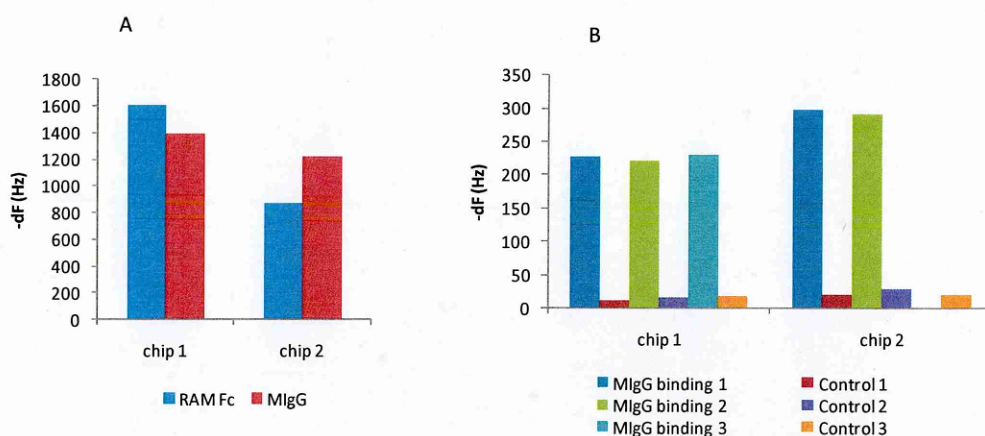


Figure 2.41. (A) Immobilisation of rabbit anti-mouse IgG and mouse IgG on amide-dextran coated QCMA-1 sensor chips. (B) 5 µg mL⁻¹ Mouse IgG binding on rabbit anti-mouse IgG (active) and mouse IgG (control) immobilised surfaces.

It is possible that the moving free dextran particles that manipulates the sample flow might have contributed to this result (as described in the previous section). To

prevent this kind of ir-reproducible results the surface fabrication procedure needs to be optimised as described in the previous section.

2.5 CONCLUSION

Surface chemistry application is a critical step of the sensor chip fabrication since the performance of the biosensor directly linked to the biomolecule immobilisation on the surface. In the current study, four different surface chemistries were investigated and the methods were optimised as SAM, PAMAM dendrimer, polymer (free radical polymerisation) and a hydrogel (CMD) surfaces.

Sensor surface chemistry application starts with the cleaning of the sensor chip surfaces, followed by the SAM deposition procedure and finishes with the quality control experiments. Plasma cleaning was chosen as an effective way of removing impurities from the gold sensor surface and this result was proven with contact angle measurements of a water droplet. The cleaned sensor chips were incubated in an ethanolic solution of alkanethiol (in this case MUDA) overnight for SAM formation on the sensor surface. And finally the formed monolayer was characterised by a cyclic voltammogram using potassium ferricyanide solution and a surface coverage of ca. 0.97 was obtained showing a very compact SAM formation on the sensor surfaces. This was ideal for the development of the cancer marker sensor platform and will be described in chapter 3.

Later PAMAM dendrimer layer was investigated as a sensing layer and found that dendrimer surface did not increase the capacity of the sensor surface if used for QCM biosensor with the current format (NeutrAvidin capture assay on biotinylated dendrimer surfaces); however protein binding capacity of the sensor increased 1.5

times if it was used for an SPR biosensor. Generation 4 dendrimer does not have a very rigid layer especially if it is immobilised on 10% or more diluted MUDA monolayer. This might have resulted in not detecting higher responses, although, the surface area of the sensor chip had increased and more protein binding occurred. In the case of SPR sensing, by the use of dendrimers immobilised on 10% MUDA the NeutrAvidin binding increased 1.3 times and by using dendrimers immobilised on 1% MUDA the NeutrAvidin binding increased 1.5 times with respect to a flat biotin layer. In short, dendrimer monolayers can be used to increase the sensor surface capacity 1.5 times for SPR sensor chips.

As an alternative surface chemistry, a polymer layer called UVlink was fabricated on sensor chips using free radical polymerisation method. Several UVlink polymer surfaces were fabricated on QCMA-1 sensor chips and used for biomolecule immobilisation and further binding assays. The results indicated that polymer surface was not suitable for QCMA-1 sensor chips. The QCM device is very sensitive to the rigidity of the polymer surface grafted on the sensor surface. It was very difficult to obtain a rigid surface (that was required to obtain a good QCM sensing) while increasing the pore size of the polymer for improving biomolecule immobilisation capacity. The developed polymer layer was also tested using an SPR biosensor. Visible and uniform polymer surfaces were fabricated on SPR sensor chips having different surface properties (flat or uniformly patterned surface) and biological tests were performed using Biacore 3000 instruments. The polymer surface has similar antibody immobilising but higher antigen binding capacity with respect to MUDA coated SPR chips; higher immobilisation capacity but slightly lower antigen binding capacity with respect to a Biacore C1 sensor chip. The main advantage of polymer

coated sensors with respect to MUDA and C1 sensor chips is the longer shelf life. While the shelf life of MUDA or C1 sensor chips are around 4 to 6 months maximum, theoretically if stored at the right conditions and depending on the polymer type the shelf life of our polymer surface is expected to be longer. Additionally different monomers (such as biotin or NTA functionalised) can be utilised to change the functional group of the polymer to achieve different type of surfaces. A patent application was filed for the work in July 2009 (Y. Uludag and I. E. Tothill, High capacity polymer surface for immobilisation of biomolecules', Date Lodged: 10 July 2009, GB0911634.4) and this was re-filed in July 2010.

Additionally a study was performed to write an operating procedure (SOP) for the fabrication of carboxymethyl-dextran (CMD) sensor chips to be used for SPR biosensors. Two different fabrication methods so-called amide-dextran and epoxy-dextran methods were applied and both methods resulted in a CMD surface on sensor chips. A functional immunoassay was applied to the fabricated Biacore 3000 and SPR2 sensor chips to investigate the performance of the coatings. It was observed that the functional assay responses obtained from the epoxy-dextran coated Biacore 3000 sensor chips and purchased CM5 sensor chips were close to each other but the surface fabrication procedure need to be improved to achieve the same antigen binding results. The same protocol was applied to SPR2 sensor chips and in general the functional assay responses were lower with respect to the responses obtained from Biacore 3000 sensor chips. This could be related to the size of the SPR2 sensor chips which might have influenced the coating process. Additionally the flow cell size of SPR2 instrument is larger than the Biacore 3000 instrument and in-efficient washing of the sensor chip after dextran coating cause flow disturbances which

might have influenced the results. It is expected to obtain better results from the dextran coated SPR2 sensor chips after the washing steps of the dextran coating procedure was improved. As the molecular weight of the CMD used for amide-dextran (MW 10,000 – 20,000) sensor coatings were lower in comparison to the epoxy-dextran (MW 500,000) method, the immobilisation of antibodies to this sensor surfaces produced lower responses with respect to epoxy-dextran coated sensor chips. However the antigen binding responses obtained using Biacore 3000 instrument was very close to the epoxy-dextran coated sensor surface. Again SPR2 instrument produced lower responses from the immunoassay applied on amide-dextran coated sensor chips. However the reason of this lower response could have similar reasons as described above for the epoxy-dextran coated SPR2 sensor chips. In short, both amide-dextran and epoxy-dextran methods are suitable for CMD coated sensor chip fabrication.

CHAPTER III:
DEVELOPMENT OF AN AFFINITY SENSOR FOR PSA
DETECTION

3 DEVELOPMENT OF AN AFFINITY ASSAY FOR PSA DETECTION

3.1 INTRODUCTION

In this chapter, the development of a simple and rapid detection method for cancer biomarkers analysis using a quartz crystal microbalance (QCM) biosensor is described. A QCM consists of a thin quartz wafer sandwiched between a pair of electrodes was used. The mode of oscillation depends on the cut and geometry of the quartz crystal. Mass addition or loss on the sensor surface results in frequency change and hence measuring molecular interactions that occur on the sensor surface without the need of a label. By employing gold (Au) nanoparticles in the assay design, it is possible to increase the mass due to antigen binding and further enhance the sensitivity of the assay (Cooper and Singleton, 2007; Fei et al., 2007). However, the frequency is also affected by viscosity change of the assay media and charge of the interaction molecules (Janshoff et al., 2000). Therefore, care needs to be taken to subtract the controls and extract the real response due to specific molecular interactions. The sensitivity enhancement using nanoparticles has been usually achieved by modifying a secondary antibody to the nanoparticles and performing a sandwich assay.

In this work an investigation was performed to develop and optimise an immunoassay to detect biomarkers in human serum on the QCMA-1 sensor chip. To minimise the matrix effect of human serum, the addition of detergent, salt and other additives to the buffer solution was investigated. PSA and PSA-ACT complex were used as the cancer biomarkers for detection and an immunoassay was developed and performed in buffer and in human serum. QCMA-1 is a prototype instrument therefore to validate the results

the same assay was repeated using a commercially available Biacore 3000 instrument in order to compare the sensitivity and validate the assay.

3.2 MATERIALS AND INSTRUMENTATION

Phosphate buffered saline (PBS, 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4) tablets, bovine serum albumin (BSA), ethanolamine, human serum, N-hydroxysuccinimide (NHS) and tween 20 were purchased from Sigma-Aldrich (Poole, UK). 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) was purchased from Pierce-Thermo Scientific (Cramlington, UK). Mouse IgG was obtained from Stratech Scientific Ltd (Newmarket, UK). 20nm Au nanoparticles purchased from BBInternational (Cardiff, UK) and 40 nm Au nanoparticles were obtained from Diagnostic Consulting Network (CA, USA). Dextran (MW 250 000) was provided from Acros Organics BVBA (Geel, Belgium). PSA, PSA detection and capture antibodies were bought from AbD Serotec (Kidlington, UK). Complexed PSA and free PSA antibodies were purchased from Alpha Diagnostics (TX, USA) and BiosPacific (CA, USA). PSMA antigen and antibody were obtained from Abcam (Cambridge, UK).

To visualise the Au nanoparticles a scanning electron microscope (SEM) (SFEG XL30, FEI, Hillsboro-Oregon, USA) was used. For AFM measurements Dimension 3000 SPM instrument (Veeco Instruments Ltd., Cambridge) was used with silicon probes (type: PPP-NCH-50).

A fully automated QCMA-1 (Sierra Sensors GmbH, Hamburg, Germany) and a Biacore 3000 (GE Healthcare, Uppsala, Sweden) devices were employed as the biosensor platforms for the development of the PSA sensor. Bare gold QCMA-1 and Biacore 3000 bare gold (SIA Kit Au) and C1 sensor chips were used for the study. The QCMA-1

sensor chips (20 MHz) possess two sensing spots each, enabling the measurement of active and control sensor surfaces simultaneously. Biacore sensor chips possess four sensing spots each, enabling the measurement of 3 active and 1 control sensor surfaces simultaneously. QCMA-1 and Biacore sensor chips and the continuous flow systems used to deliver buffer and samples to the sensing spots are shown in Figure 3.1 and Figure 3.2.

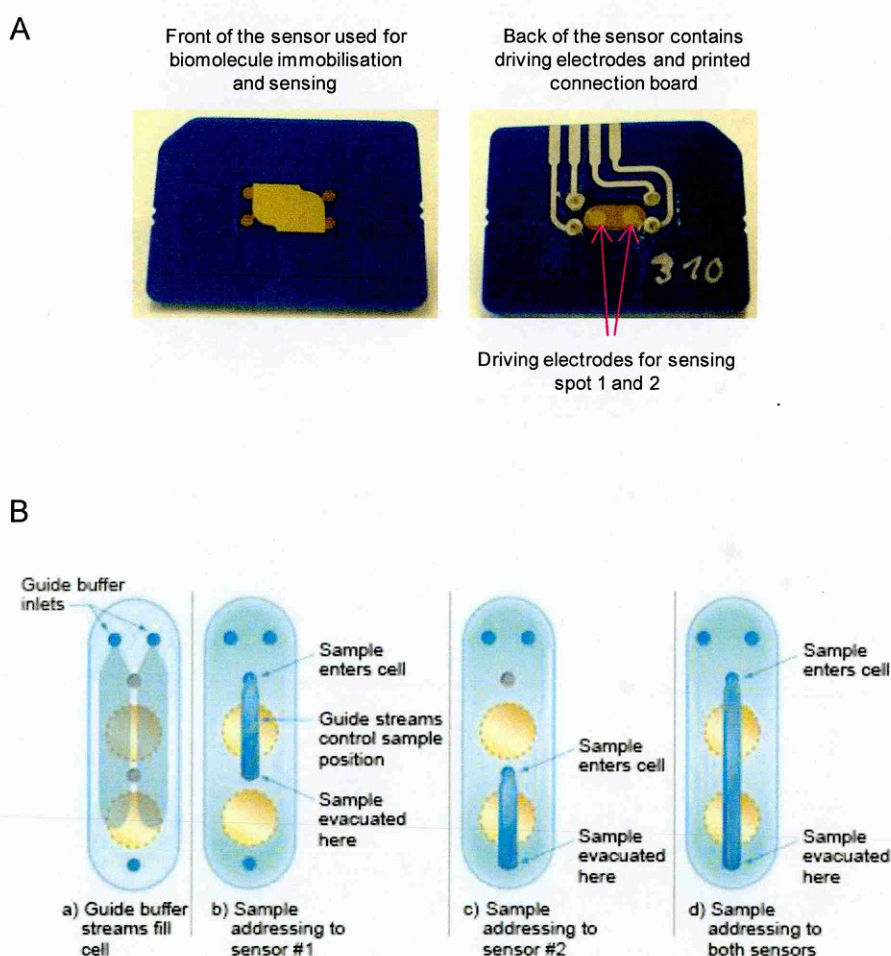


Figure 3.1. A) The front and back of a QCMA-1 affinity sensor chip. B) Sample addressing in a two sensor spot flow cell of a QCMA-1 affinity sensor chip using hydrodynamic isolation technique (www.sierrasensors.com).

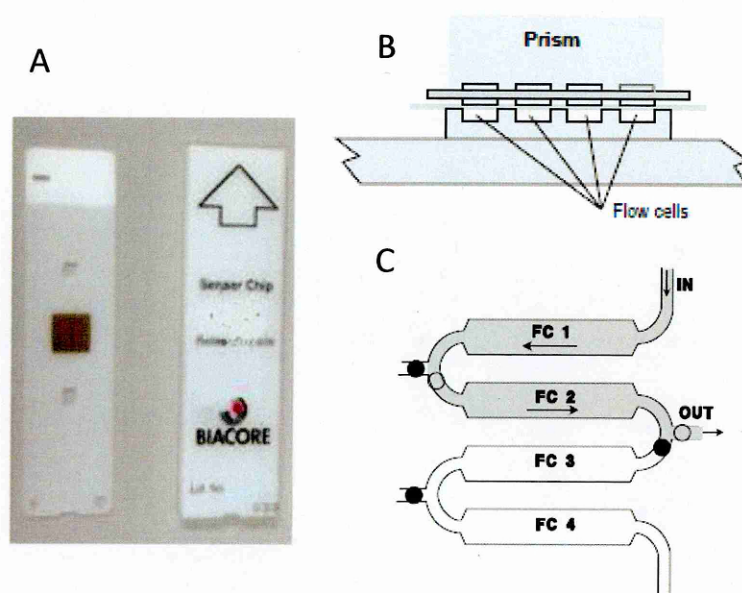


Figure 3.2. A) A Biacore 3000 sensor chip. B) An integrated fluidic cartridge creates four flow cells (C) enabling simultaneous measurement of four surfaces (Biacore, 2003a).

3.3 METHODS

3.3.1 Modification of Au nanoparticles with Anti-PSA detection antibody

10 μl of 1 mg mL^{-1} PSA detection antibody was added to the pH-adjusted Au nanoparticle (20nm or 40 nm) solution (1 mL) and incubated for an hour at room temperature on a shaker. A 100 μl of 10 mg mL^{-1} BSA was added to the solution to ensure that the Au nanoparticles are all coated with antibody or protein. After 20 minutes of incubation the solution was centrifuged for 30 minutes at 10 000 rpm at 4°C (Biofuge pico centrifuge, Heraeus Instruments). After spinning, the antibody modified nanoparticles were recovered and re-suspended in 100 μl of PBS/T. The concentration of nanoparticles was determined by a spectrophotometer at 520 nm wavelength (WPA Lightwave UV/visible Spectrophotometer, WPA). According to the information obtained from the manufacturer (BBInternational, Cardiff, UK) the optical density (OD)

of 1 at 520 nm wavelength corresponds to 7×10^{11} (20nm) nanoparticles mL^{-1} and 9×10^{10} (40 nm) nanoparticles mL^{-1} . The antibody modified nanoparticles were diluted 100 times and pipetted into a cuvette for absorbance measurements at 520 nm. Equation 3.1 was used to determine the concentration of the Au nanoparticles (C_{Np}) that are modified with antibody.

$$C_{\text{Np}} = \text{Measured OD} \times C_{\text{Np at OD 1}} \times 100 \quad \text{Eq. 3.1}$$

The antibody modified Au nanoparticles were then stored in the fridge (4°C) until use. Scanning electron microscope (SEM) images were taken using a FEI, XL 30-SFEG (Eindhoven, Netherlands) instrument.

3.3.2 Antibody immobilisation to the sensor surface

Sensor surfaces were prepared by immobilising PSA capture antibody (produced from mouse) on the active sensor surface and mouse IgG on the control sensor surface on a QCMA 1 and Biacore sensor chips. The operating temperature of the assays was 25°C and the flow rate of the running buffer was $80 \mu\text{l min}^{-1}$ for QCMA-1 and $10 \mu\text{l min}^{-1}$ for Biacore 3000 assays. QCMA-1 and Biacore 3000 sensor chips were coated with MUDA as described in Section 2.3.1 after a plasma cleaning process and coated chips were then stored in the fridge until their use. As QCMA-1 sensor chips contain 2 sensing spot, one was used as active for PSA capture antibody immobilisation and the other spot was used as control and mouse IgG was immobilised. Biacore 3000 sensor chips have 4 flow channels and while one channel was used as control other three was used as active. The same sensor surface preparation and antibody immobilisation protocols were applied for both QCMA-1 and Biacore 3000 assays. The assay procedure was programmed to QCMA-1 control software as a batch list and a method containing script language was written for the Biacore 3000 experiments. The immobilisation was achieved using

conventional amine coupling chemistry (Staros et al., 1986). Running buffer used for immobilisation was degassed PBS. Both QCMA-1 and Biacore 3000 sensor chip surfaces were first activated with a 1 : 1 mixture of 400 mM EDC and 100 mM NHS, prepared in 0.22 μm filtered deionised water, and mixed immediately prior to use (final concentrations: 200 mM EDC and 50 mM NHS). EDC-NHS mixture was injected simultaneously across the sensor surfaces for 3 min. A 30 $\mu\text{g mL}^{-1}$ anti-PSA antibody (in 10 mM sodium acetate buffer, pH 5.5) to the active channel(s) and then 15 $\mu\text{g mL}^{-1}$ mouse IgG (in 10 mM sodium acetate buffer, pH 5.5) to the control channel were injected for 3 minutes. The sensor surfaces were then blocked with 50 $\mu\text{g mL}^{-1}$ BSA in PBS for 3 minutes. Non-reacted NHS esters were capped with 1 M ethanolamine, pH 8.5 for 3 minutes. The frequency or response changes were recorded 2 minutes after the protein injection was completed. Schematic of the sensor surface preparation was shown in Figure 3.3.

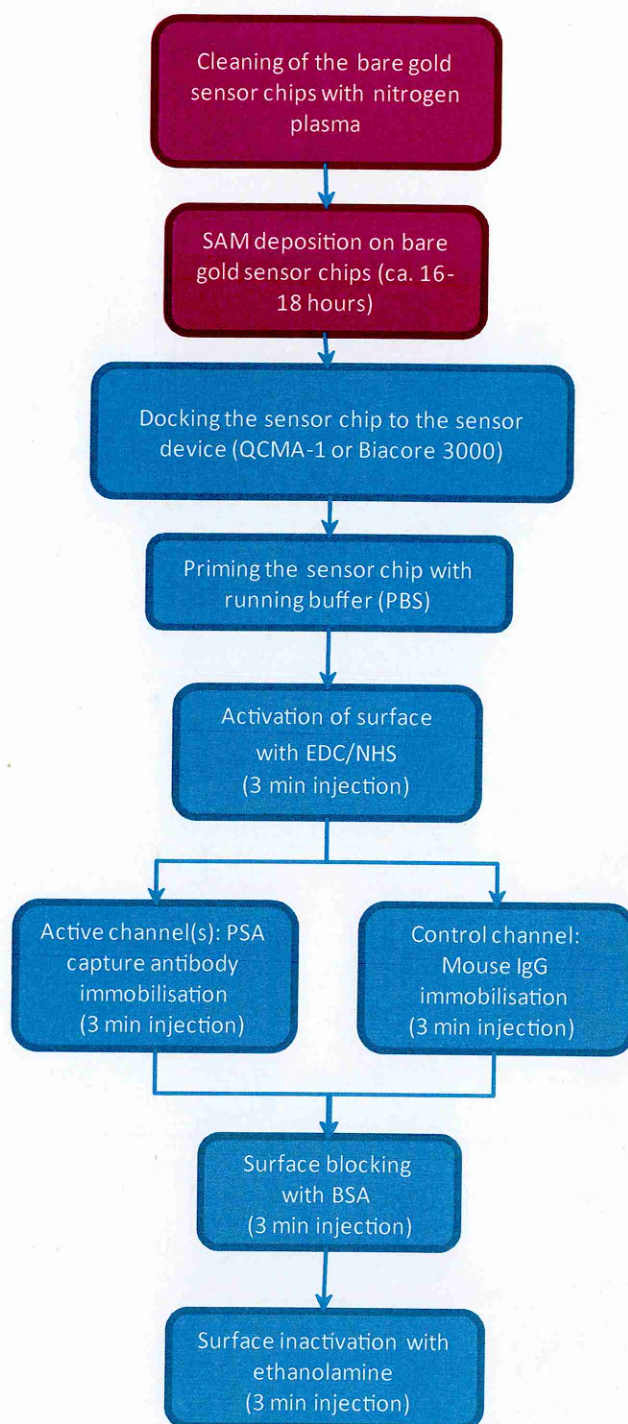


Figure 3.3. Schematic of sensor surface preparation prior to PSA detection assay.

3.3.3 Optimisation to minimise matrix interference

To optimise the sensor for PSA analysis in blood samples the effect of human serum on the assay was examined using the QCMA-1 instrument. After antibody immobilisation, the running buffer was changed to PBS buffer containing 0.005% Tween 20 (PBS/T). Human serum solution (10%) was prepared in PBS/T buffer and injected over to Mouse IgG immobilised sensor surface. After a dissociation period under running buffer flow, the surface was regenerated with the injection of 100 mM HCl (60s) and 20 mM NaOH (60s). Then 10 % human serum was prepared in PBS/T buffer containing additives at varying concentrations and each human serum preparation was injected to the Mouse IgG immobilised surface for 3 minutes. The sensor surface was regenerated between human serum applications as described above. The non-specific binding of human serum proteins on the sensor surface was determined by subtracting the frequency responses before the injection and 100s after the injection stopped. The buffer designed to minimise the non-specific binding of serum proteins were used for assays performed with QCMA-1 and Biacore 3000 instruments.

3.3.4 PSA detection assay

After the antibody immobilisation on QCMA-1 and Biacore 3000 sensor chips, PSA detection assay was performed. In serum, PSA is found either in free form or as a complex with alpha 1-antichymotrypsin (ACT) (ACT-PSA, MW 96 kDA). Total PSA (tPSA) refers to the PSA in both forms (PSA and ACT-PSA complex). In the current study to prepare tPSA 1 to 1 mixture of PSA and ACT-PSA was used. PSA or tPSA was diluted at specified concentrations (0.29 to 5000 ng mL⁻¹) in PBS/T buffer containing 5 µg mL⁻¹ BSA or 10% to 75% human serum in PBS/T buffer containing additives. These solutions were then injected over the PSA capture antibody and mouse IgG immobilised surfaces for 3 or 5 min to allow binding interactions. The frequency

changes (QCMA-1) or response changes (Biacore 3000) due to PSA/tPSA binding were recorded at 180 s after the injection started. After the binding of PSA/tPSA either surface was regenerated by injection of 100 mM HCl (1 min, 80 μ l) or the assay was continued to perform a sandwich assay. A schematic of the PSA direct assay performed using QCMA-1 and Biacore 3000 devices is shown in Figure 3.4.

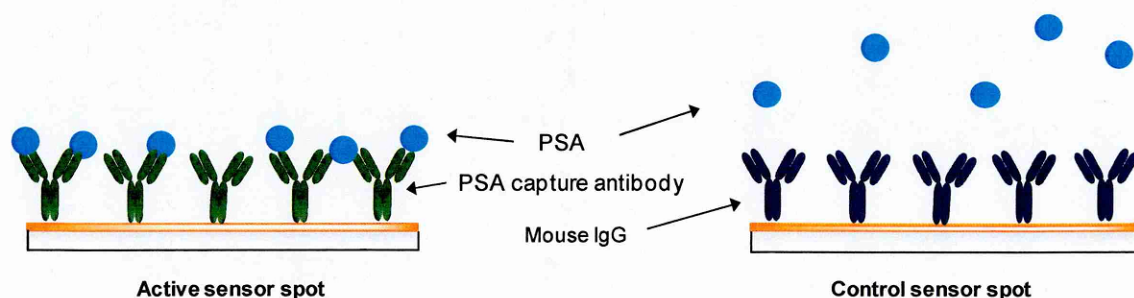


Figure 3.4. Schematics of PSA direct assay.

After the binding of PSA/tPSA to the sensor surface, $1.5 \mu\text{g mL}^{-1}$ PSA detection antibodies or PSA detection antibody modified Au nanoparticles were injected on the sensor surface for 3 or 5 min (240 or 400 μ l). After 3 min dissociation period under running buffer flow, surfaces were regenerated by injection of 100 mM HCl (1 min, 80 μ l) (and additional 20 mM NaOH (1 min, 80 μ l) was injected if PSA spiked human serum was used). The frequency changes due to PSA detection antibody binding were recorded 3 min after the injection started. A schematic of the PSA sandwich assay is shown in Figure 3.5.

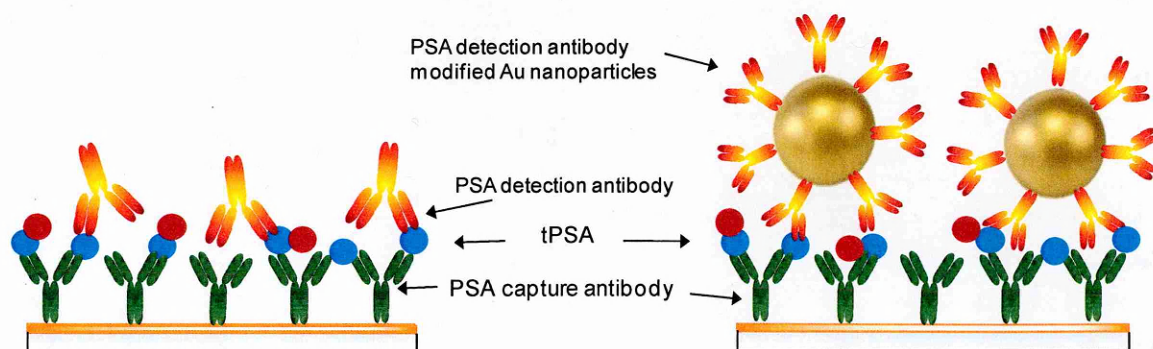


Figure 3.5. Schematics of PSA sandwich assay using PSA detection antibody or antibody modified Au nanoparticles.

The limit of detection (LOD) was calculated as the signal obtained from the PSA concentration that is equivalent to the 3 times the standard deviation of the signals obtained from the blank standards. The coefficient of variation (CV) was calculated according to the equation 3.2 as the ratio of standard deviation (σ) to the mean (μ) (Brooks, 2001).

$$\% CV = \frac{\sigma}{\mu} \times 100 \quad (\text{Eq. 3.2})$$

Higher CV indicates greater dispersion in the variable; therefore smaller CV values are desired for the assay results.

3.3.5 Ethical Approval

Thirty healthy male participants (with no history of prostate disease or current urinary symptoms) were recruited at Cranfield University, and favourable ethical approval was obtained from the Cranfield Health Ethics Research Committee. Sixty consecutive patients who attended the TRUS-prostate biopsy sections at the Primrose cancer unit, Bedford hospital, were recruited, and ethical approval was obtained from the Bedfordshire Research Ethics Committee. All the participants gave their informed consent before sample collection. All the documents for ethics application and the approval letters are contained in appendix B. The study on clinical samples was designed to sample blood from patients and controls. Clinical data including patient's age, ethnicity, PSA at referral, Gleason Score, Clinical stage and Prostatic volume were extracted from clinical notes.

3.3.6 Sample Collection and Processing

A 5mL of venous blood was collected into empty or K₃ EDTA tubes from each of the participants (healthy males, control group, n=30) and patients (n= 60) using standard venom puncture techniques. Plasma or serum was harvested after centrifugation at 1800 g for 5 minutes, and stored at -80 °C until required for use.

3.4 DEVELOPMENT OF A QCM AFFINITY SENSOR FOR PSA ANALYSIS

3.4.1 Characterisation of the modified Au nanoparticles using SEM

“Although the precise size differs from antibody to antibody, it has been estimated to be 15-20 nm long by 6 to 15 nm wide” (Kreier, 2002). Using these dimensions the footprint of an antibody can be calculated as $2.36 \times 10^{-16} \text{ m}^2$ (length estimated 20 nm, width estimated 15 nm). The approximate number of antibodies on a nanoparticle can be calculated with an assumed packing density of 0.907 (the circle packing density limit) (Schwartz, 2007) using the equation 3.2.

$$\text{number of antibodies} = 0.907 \frac{\text{nanoparticle surface area}}{\text{antibody footprint area}} \quad \text{Eq. 3.2}$$

The approximate number of antibodies that can fit on a 20 nm nanoparticle was calculated as 5 and on a 40 nm nanoparticle calculated as 17 using the equation 3.2. Both 20 nm and 40 nm Au nanoparticles were modified with detection antibody and SEM images were taken before and after antibody modification. For 20 nm Au nanoparticles, the sizes of the nanoparticles were measured as 16 to 20 nm before modification and after antibody modification the sizes were measured between 22 to 31 nm (Figure 3.6). The size differences between the modified and non-modified nanoparticles were between 6 to 15 nm, not more than the width of one antibody. Therefore, although theoretically 5 antibodies can fit on a 20 nm nanoparticle, SEM image indicated that one antibody was adsorbed on one 20 nm nanoparticle. For 40 nm Au nanoparticles, SEM image revealed that the sizes of nanoparticles vary between 20 to 42 nm and this prevented the comparison of uncoated and antibody modified nanoparticles by just looking at the SEM image. The uncoated Au nanoparticles do coagulate in salt solution therefore the existence of antibody coating on the

nanoparticles also were confirmed by addition of salt solution to the Au nanoparticle suspension. The salt added antibody-modified Au nanoparticle suspension did not coagulate or changed the colour, proving the successful antibody adsorption.

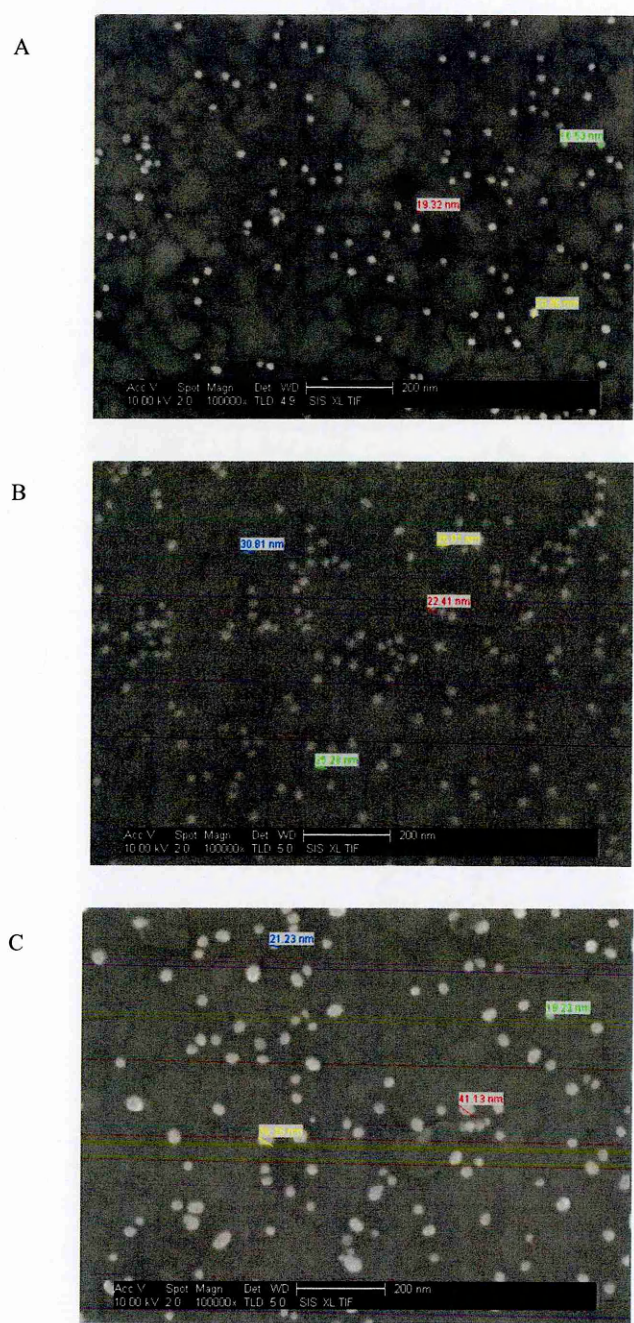


Figure 3.6. SEM images of Au nanoparticles. The sizes of the 20 nm nanoparticles were measured as 16 to 20 nm before modification (A) and after antibody modification the sizes were measured between 22 to 31 nm (B). SEM image of 40 nm Au nanoparticles showed that the size of nanoparticles have a larger size variation that was between 20 to 41 nm (C), and this made it difficult to compare uncoated and antibody modified nanoparticles by just looking at the SEM images.

3.4.2 Optimisation of sensor signal

Bare gold QCMA-1 sensor chips were employed in this work as the sensor platform for PSA detection. Each chip consists of two sensing array for active and control sample testing. The modification of the chips using self assembled monolayer (SAM) was carried out on the sensor surface. Atomic Force Microscopy (AFM) images of a sensor chip were taken before and after the SAM coating (Figure 3.7 and Figure 3.8). The results indicate that SAM modification caused a change of the root mean square (rms) roughness from 5.5 nm to 3.1 nm due to surface coating using MUDA. The quality of SAM formed on QCMA-1 sensor chips were also tested with cyclic voltammetry as described in Section 2.4.1 and the results indicated a very compact layer formation on the sensor surface. The AFM images did confirm this finding and enabled the visualisation of the monolayer prepared.

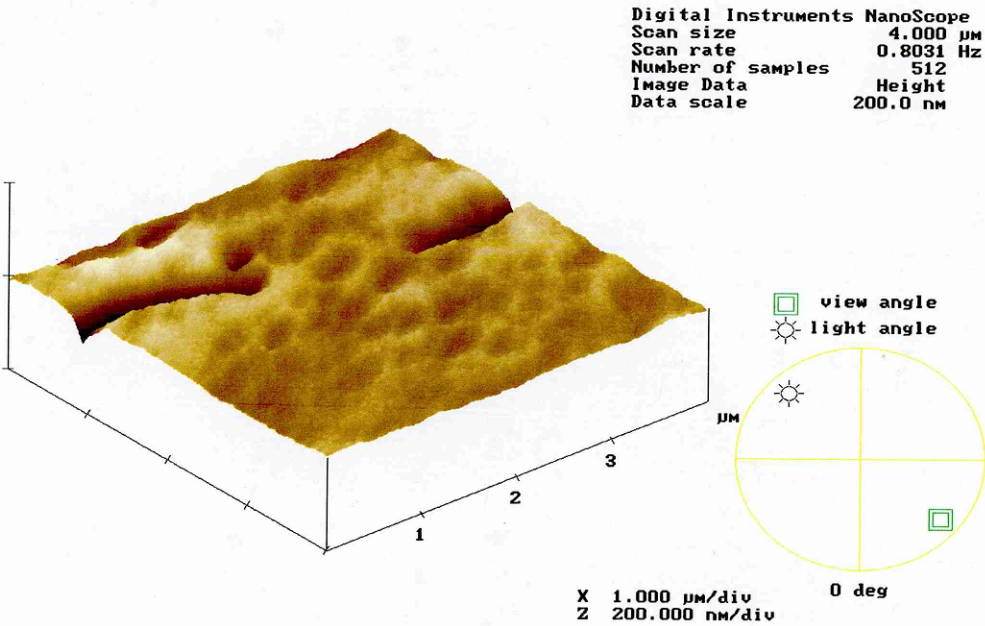


Figure 3.7. The AFM image of a bare gold QCMA-1 sensor chip.

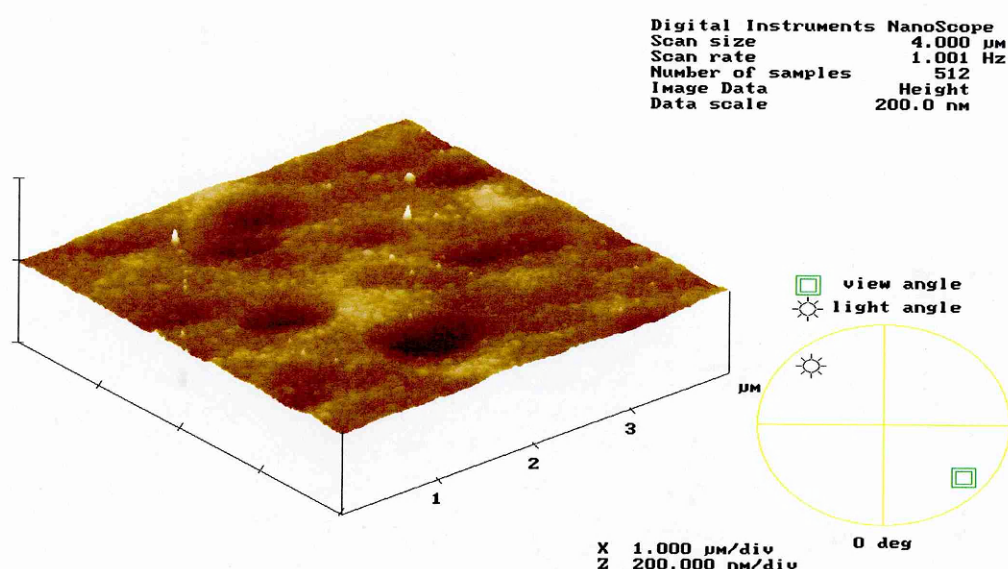


Figure 3.8. The AFM image of a SAM coated QCMA-1 sensor chip.

The SAM coated sensor chips was first docked to the QCMA-1 instrument and primed with running buffer to wet the sensor chip and continues buffer flow ($80 \mu\text{l min}^{-1}$) was started. Anti-PSA capture antibody and mouse IgG were then immobilised on the active sensor array and the control sensor array respectively using conventional EDC-NHS chemistry (Lšfas and McWhirter, 2006). A 3 minutes injection of antibodies was sufficient to achieve signal to reach equilibrium; therefore, the immobilisation time was kept at 3 minutes for the assay. Three different concentrations ($15\text{-}30$ or $50 \mu\text{g mL}^{-1}$) were used for antibody immobilisation and from these $30 \mu\text{g mL}^{-1}$ was chosen as the optimum concentration to immobilise the antibodies. There was not much difference of immobilisation response when $50 \mu\text{g mL}^{-1}$ or $30 \mu\text{g mL}^{-1}$ of antibody was used, additionally the cost of consumables wanted to be kept to a level therefore $30 \mu\text{g mL}^{-1}$ was chosen as the standard concentration for antibody immobilisation. The effect of EDC/NHS mixture injection time was also investigated by performing assays with 3 minutes or 7 minutes of EDC/NHS mixture injection. No difference was found between the antibody immobilisation levels by elongating the EDC/NHS injection and hence 3

minutes was chosen for the assay. The antibody injection time was kept as 3 minutes to minimise the consumption of the reagent. With 3 minutes EDC/NHS injection time and $30 \mu\text{g mL}^{-1}$ antibody concentration, anti-PSA capture antibody immobilised sensors produced an average frequency change of $380 \pm 38 \text{ Hz}$ and mouse IgG immobilised sensors produced a frequency change of $520 \pm 15 \text{ Hz}$ (Figure 3.9). The immobilisation levels of antibodies depend on both the concentrations of antibodies used and the solution content of the purified antibodies. Although the antibodies used are both mouse IgG antibodies, both are prepared by different companies and purified using different methods. Therefore, different levels of immobilisation were obtained using anti-PSA capture antibody and mouse IgG antibody.

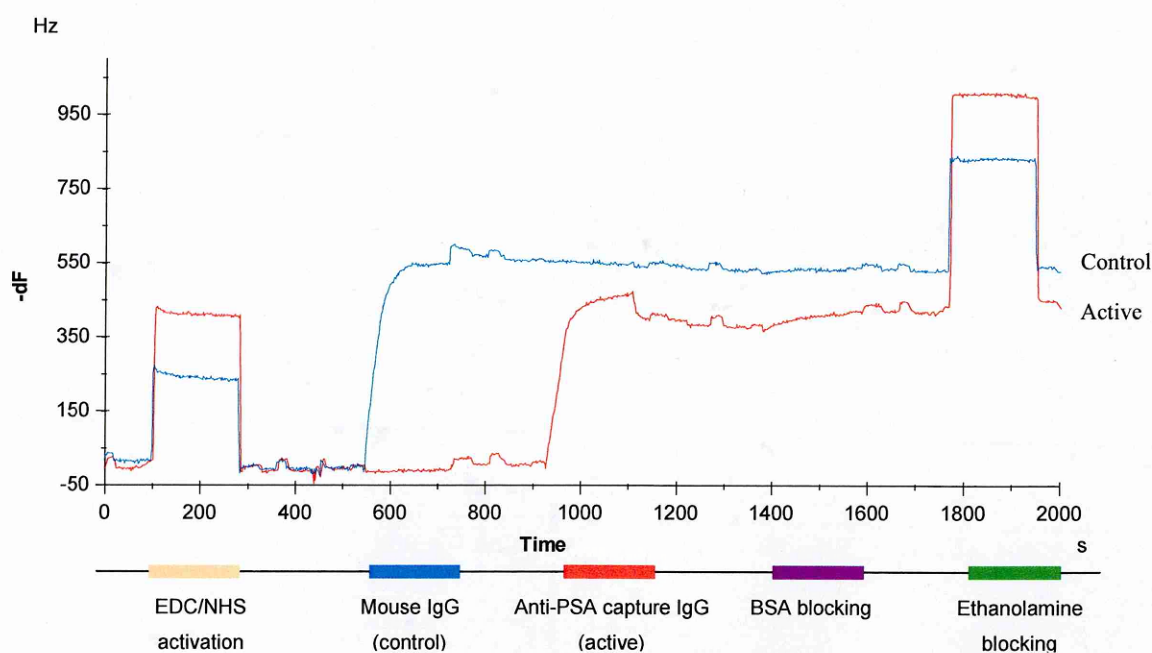


Figure 3.9. Immobilisation of anti-PSA capture antibody and mouse IgG on the active and control sensor spots of a QCMA-1 sensor chip.

The PSA non-specific binding (diluted in PBS/T buffer containing $5 \mu\text{g mL}^{-1}$ BSA) to the control sensor array was then examined by conducting binding tests. The results show that the control sensor surface did not give any frequency change even if the

highest concentration of PSA ($5 \mu\text{g mL}^{-1}$) was used (Figure 3.10). To examine the non-specific binding of BSA on the PSA capture antibody immobilised surface, BSA ($5 \mu\text{g mL}^{-1}$) was injected for 3 min and the non-specific binding of BSA to the surface was detected as $4 \pm 1 \text{ Hz}$ ($n = 3$, data not shown).

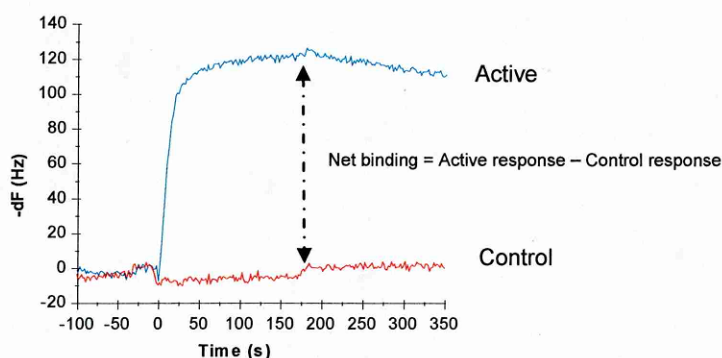


Figure 3.10. Binding of $5 \mu\text{g mL}^{-1}$ PSA on to anti-PSA capture antibody immobilised surface (blue) and mouse IgG immobilised surface (red) is shown. Non specific binding of PSA on the mouse IgG surface does not result in any frequency response.

3.4.3 Direct PSA assay

The calibration curve obtained with PSA binding (3 min) to the PSA capture antibody immobilised surface in a concentration range between 4.7 to 5000 ng mL⁻¹ is shown in Figure 3.11 and Figure 3.12. The detection limit of the PSA was found as 18.8 ng mL⁻¹, which was well above the clinical PSA level in serum. PSA binding response curves were then fitted to 1:1 Langmuir binding model to determine the binding association and dissociation rates (Rich and Myszk, 2009), from which K_D value was calculated as 5.56×10^{-10} M and R_{max} as 108 Hz using BiaEvaluation Software (Biacore, GE Healthcare, Sweden) (Table 3.1). More detailed information related with the calculation of kinetics rates are described in Section 3.6. Karlsson *et al.* calculated the affinity of PSA antibody towards PSA as 3.3×10^{-9} M (antibody from Fitzgerald Industries Int., clone M212091) (Karlsson *et al.*, 2006). Katsamba *et al.* calculated the affinity as 1.1×10^{-9} M (antibody from Fitzgerald Industries Int., clone M612166) (Katsamba *et al.*, 2006).

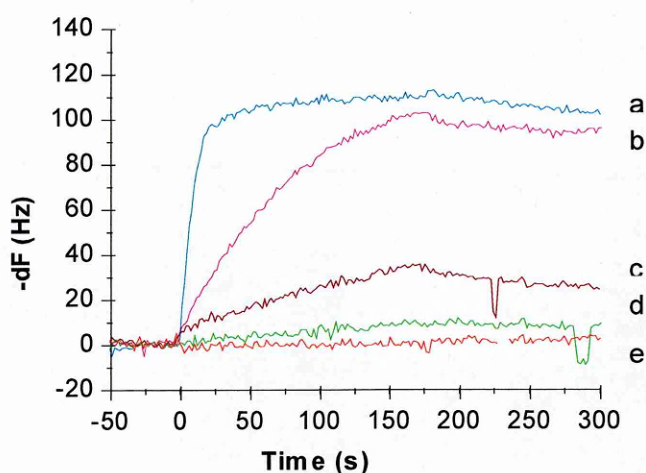


Figure 3.11. QCM sensor response for different concentrations of PSA samples (3 min injection). After each sample injection, the surface was regenerated with a 1 min injection of 100 mM HCl (data not shown). (a) 5000 ng mL⁻¹, (b) 312 ng mL⁻¹, (c) 78 ng mL⁻¹, (d) 18.8 ng mL⁻¹ (all control subtracted data) and (e) the response obtained from the binding of 5000 ng mL⁻¹ PSA to Mouse IgG immobilised control surface.

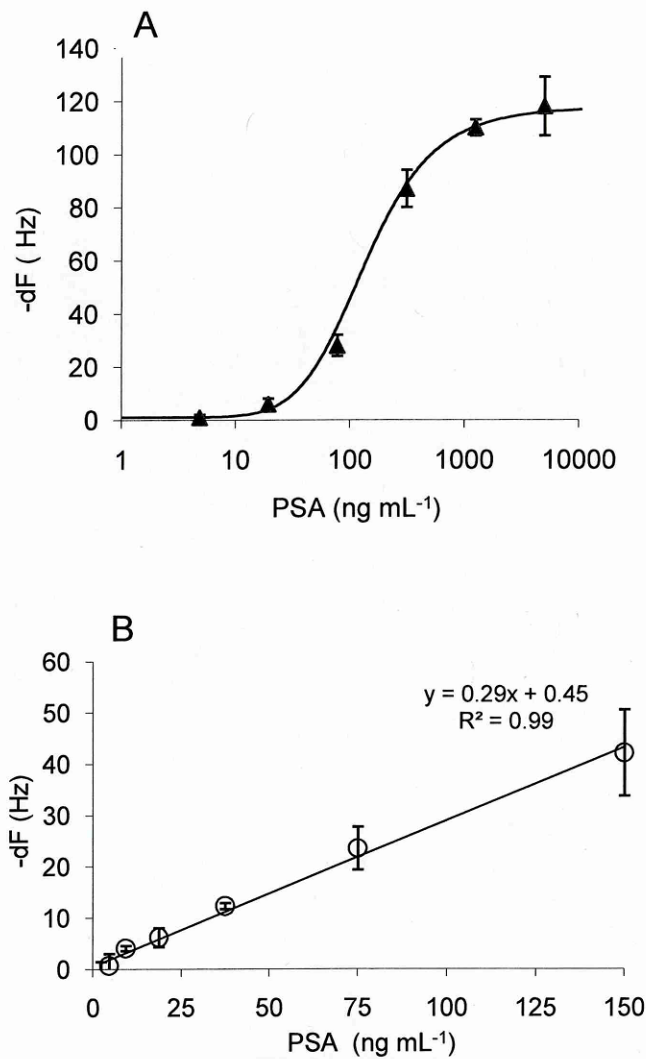


Figure 3.12. The standard curves (A - log scale; B - linear scale) obtained for PSA direct binding. PSA was prepared in PBS/T buffer containing 5 $\mu\text{g mL}^{-1}$ BSA and diluted in 1:2 dilution steps.

Table 3.1. PSA direct assay results of kinetic calculations.

$K_D = 5.56 \times 10^{-10} \text{ M}$
$K_a = 1.23 \times 10^{+06} \text{ M}^{-1} \text{ s}^{-1}$
$K_d = 6.83 \times 10^{-04} \text{ s}^{-1}$
$R_{\text{max}} = 108 \text{ Hz}$

3.4.4 Sandwich assay with anti-PSA detection antibody

To enhance the sensor signal and improve the sensitivity of the method, a sandwich assay approach was developed employing PSA detection antibody. The calibration curve was obtained for the sandwich assay in a concentration range between 150 to 2.3 ng mL⁻¹ that is relevant for prostate cancer diagnosis (Figure 3.14 and Figure 3.14). This assay resulted in 4.7 ng mL⁻¹ PSA as detection limit which is four folds more sensitive than the direct assay. The experiments have shown that the chosen anti- PSA antibody has good affinity for a successful immunoassay and the sandwich assay resulted in a clinically relevant detection limit (4 ng mL⁻¹). Consequently experiments were performed to optimise the assay so that biomarker detection could be performed in human serum. The coefficient of variation (CV) laid between 2 to 12% across the range 9.4 to 150 ng mL⁻¹ PSA concentrations and CV was 24% for the lowest concentration detected. Small signal with a relatively high standard deviation caused high CV value when lowest PSA concentration was detected.

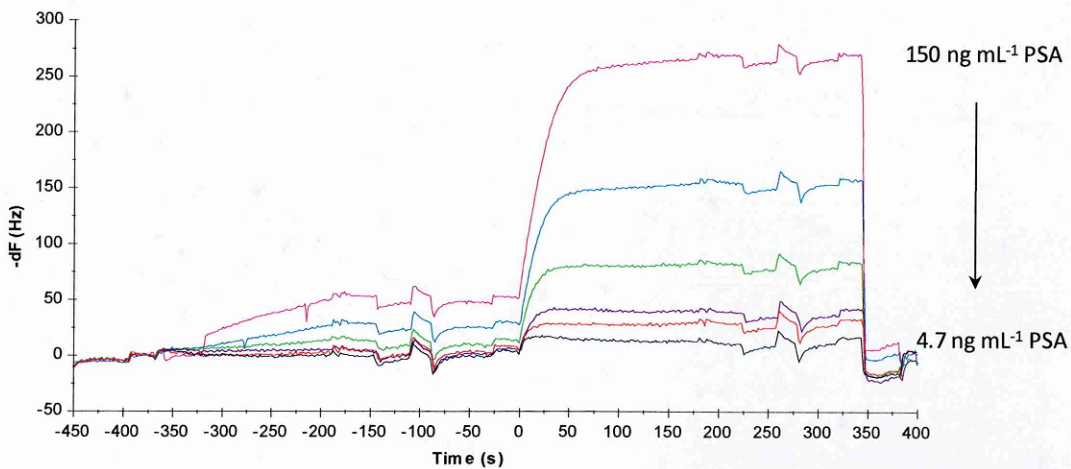


Figure 3.13. 3 min binding of PSA samples at concentrations 4.7, 9.4, 18.8, 37.5, 75 and 150 ng mL⁻¹ was followed with 3 min binding of PSA detection antibody (1.5 μg mL⁻¹). Assay was performed using a QCMA-1 biosensor.

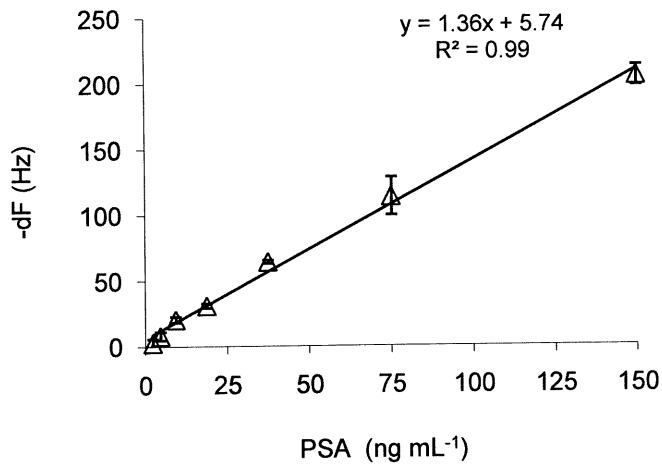


Figure 3.14. The standard curves obtained for PSA sandwich assay. 3 min binding of PSA samples at different concentrations was followed with 3 min binding of PSA detection antibody ($1.5 \mu\text{g mL}^{-1}$).

3.4.5 Buffer optimisation for human serum sample analysis

There is a significant difference in the limit of detection when buffer or clinical sample (serum) was used as the assay media. For example, although Cao *et al.* performed the PSA-ACT assay using only 10% human serum; the limit of detection changed from 10.2 to 18.1 ng mL⁻¹ (Cao et al., 2006). This change in the detection limit, when clinical samples are used, is mainly due to lower signal to noise ratio resulting from the high non-specific binding of sera proteins/antibodies to the sensor surface. This is especially noticeable when label-free biosensors are employed, and this prevents researchers from the use of high concentrations of human serum. Therefore, most analyses are conducted using concentration of human serum in the range of 10 to 50 % (Cheng et al., 2008; Kurosawa et al., 2004; Situ et al., 2008). In the current study we investigated the use of additives to minimize the adsorption of sera proteins to the sensor surface.

Initially, a 10% human serum diluted in PBS/T buffer was injected on the mouse IgG immobilised sensor surface. A 1490 Hz response was obtained from this solution and after buffer flow started, the response was reduced to 1370 Hz (Figure 3.15-trace 1).

From the results it was evident that human serum proteins caused a significant non-specific binding on the surface. To reduce this non-specific binding salt, BSA, dextran and Tween 20 were added to the buffer at varying concentrations. Increase the salt concentration increases the ionic strength of the solution and that lowers the electrostatic attraction of human serum proteins to the sensor surface. Therefore, high salt concentration was used to reduce proteins adsorption to the sensor surface. Dextran and BSA were used to help in preventing serum protein absorption. It is common to employ detergents to lower the non-specific protein binding; therefore the concentration of Tween 20 was increased in the solution (Ayela et al., 2007). These additives at

varying concentrations were added to prepare 10% human serum and injected over the mouse IgG immobilised sensor surface for 3 min to see the effect of the additives on serum non-specific binding to the sensor surface (Figure 3.15). From the several different buffers tested, the QCM responses of four of them are shown in Figure 3.15.

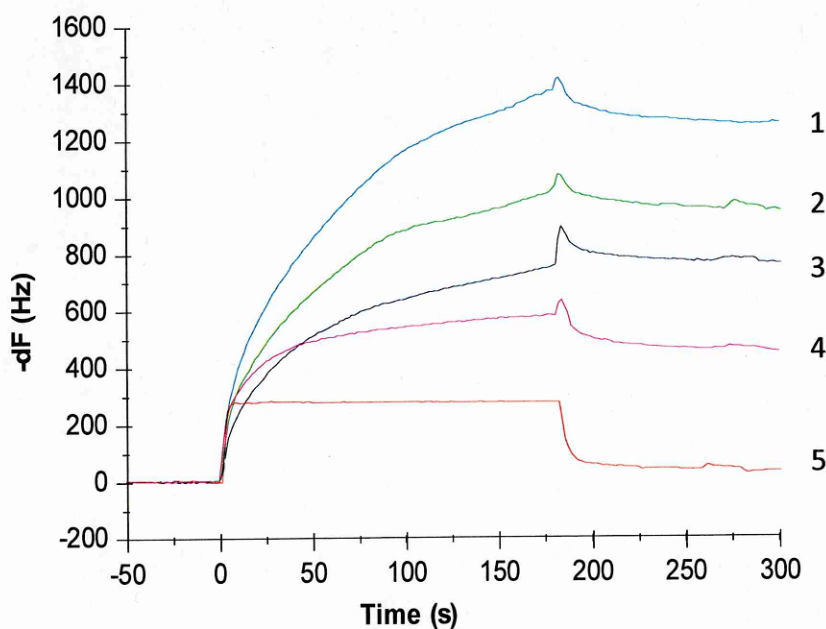


Figure 3.15. Effect of changing the buffer composition on the human serum signal. The injection of 10 % human serum in PBS/T buffer (1) and PBS/T buffers containing additives at varying concentrations (buffer 2 ($100 \mu\text{g mL}^{-1}$ BSA), 3 ($200 \mu\text{g mL}^{-1}$ BSA), 4 ($200 \mu\text{g mL}^{-1}$ BSA and 0.5% Tween), 5 ($200 \mu\text{g mL}^{-1}$ BSA, 0.5 M salt, $500 \mu\text{g mL}^{-1}$ dextran and 0.5% Tween 20) to mouse IgG immobilised sensor chip.

The lowest non-specific binding was observed when the serum is diluted in additives containing PBS/T buffer that had final concentrations of $200 \mu\text{g mL}^{-1}$ BSA, 0.5 M salt, $500 \mu\text{g mL}^{-1}$ dextran and 0.5% Tween 20 in solution. The response 180s after the injection of 10% human serum diluted in this matrix elimination buffer (matrix buffer) was found as $251 \pm 18 \text{ Hz}$ ($n = 3$) and after the buffer flow started the response was reduced to $23 \pm 12 \text{ Hz}$ ($n = 3$). As it can be seen from Figure 3.15 (trace 1), the injection of human serum in PBS/T buffer resulted in a trace that showed a curved structure

indicating the binding of sera proteins to the sensor surface and there was little dissociation after the injection stopped and buffer flow was started. Whereas when human serum diluted in the matrix buffer was injected (Figure 3.15 - trace 5), the response trace showed an instant sharp increase up to ca. 250 Hz and remained showing no curvature indicated that the bulk of this response might be due to the viscosity of the solution and hence after buffer flow the response reduced to a much lower frequency of ca. 20 Hz corresponding to adsorbed sera proteins. Figure 3.16 shows the comparison of non-specific binding results for 10%, 40% and 75% human serum diluted in the new buffer and injected to mouse IgG immobilised surface. The low non-specific binding values for these three concentrations of human serum (highest 91 ± 10 Hz for 75% human serum) indicated that this new buffer was effective even for high serum concentrations. For all the forthcoming experiments human serum was always diluted with the matrix buffer.

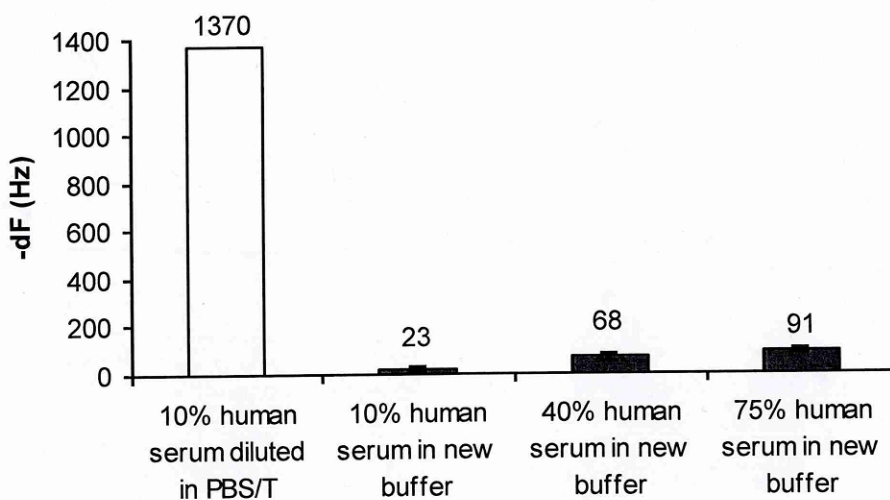


Figure 3.16. Effect of increasing human serum concentration on the sensor signal. Human serum was diluted in new formulated buffer at varying concentrations and injected (3 minutes) on mouse IgG immobilised surface.

These results showed that by applying the matrix buffer in the assay method, a 98% reduction in non-specific binding of human sera proteins was achieved. If this result is

compared to other recently published work; Situ et al. achieved 94% reduction in non-specific binding by using CM5 (carboxymethyl dextran) surface and a formulated buffer (10mM HEPES, 3mM EDTA, 0.005% Tween 20, 0.5M NaCl, 0.005% CMD-500, pH 9.0) together and Trevino et al. achieved 88% reduction in non-specific binding of serum by using another formulated buffer (PBS buffer containing 0.5M NaCl, 0.1% Tween 20, pH 8) (Situ et al., 2008; Trevino et al., 2009).

The PSA assay was then performed using 20 ng mL^{-1} PSA spiked in 10% and 40% human serum diluted in matrix buffer. When the results were compared with PSA assay in PBS/T buffer, it was seen that both direct and sandwich PSA assay results in buffer or in spiked in 10% human serum (diluted in matrix buffer) were very close to each other and within the standard deviation (Figure 3.17). However, as the human serum concentration increased to 40%, the response from direct assay was eliminated but still there was a response from the injection of PSA detection antibody (sandwich assay). Although this result was lower than the assay in PBS/T buffer (or assay in 10% human serum) still good enough to perform the assay in high human serum concentration. An example to direct and sandwich assay response of PSA spiked in 10% human serum is shown in Figure 3.17.

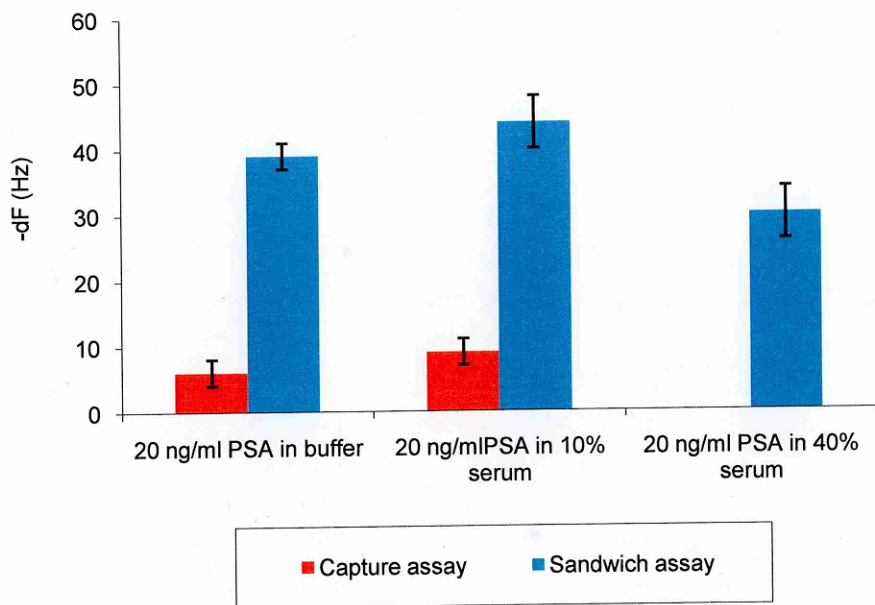


Figure 3.17. Direct and sandwich PSA assay results obtained in buffer, 10 % human serum and 40 % human serum using QCMA-1 biosensor. Each error bar represents standard deviation from three replicates.

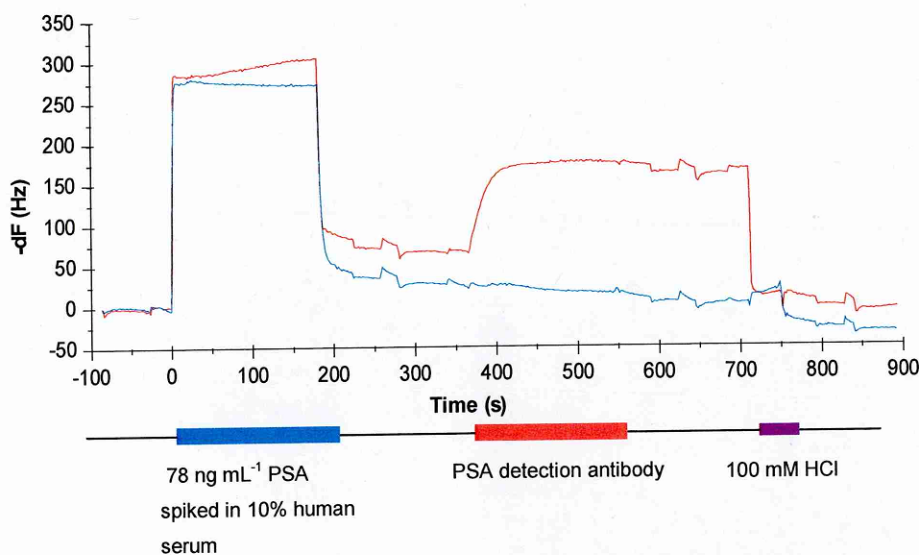


Figure 3.18. Direct and sandwich assay with 78 ng mL⁻¹ PSA spiked 10% human serum on active (a) and control (b) sensing spots of a QCMA-1 biosensor chip.

3.4.6 Detection of tPSA in 75% human serum

A calibration curve was obtained for the sandwich assay in a concentration range between 2.3 to 150 ng mL⁻¹ tPSA spiked in 75% human serum. The non-specific binding of PSA detection antibody after the injection of 75% human serum (without spiked PSA) was 2 ± 1 Hz, if assay performed on mouse IgG immobilised surface and 10 ± 3 Hz if assay performed on PSA capture antibody immobilised surface. The PSA detection antibody binding responses were obtained after the subtraction of the non-specific binding response. The detection limit obtained for this assay was 9.4 ng mL⁻¹ (Figure 3.19 - trace a). However this result was not sensitive enough for PSA detection at the required levels in serum. To enhance the sensitivity further 40 nm Au nanoparticles were modified with PSA detection antibody and sandwich assay was performed to detect tPSA. The use of Au nanoparticles lowered the detection limit down to 0.29 ng mL⁻¹ (Figure 3.19 - trace b), which is well below the required limit of detection. A sample PSA detection assay using detection antibody modified 40 nm Au nanoparticles are shown in Figure 3.20 and the responses obtained from concentration series tested were shown in Figure 3.21.

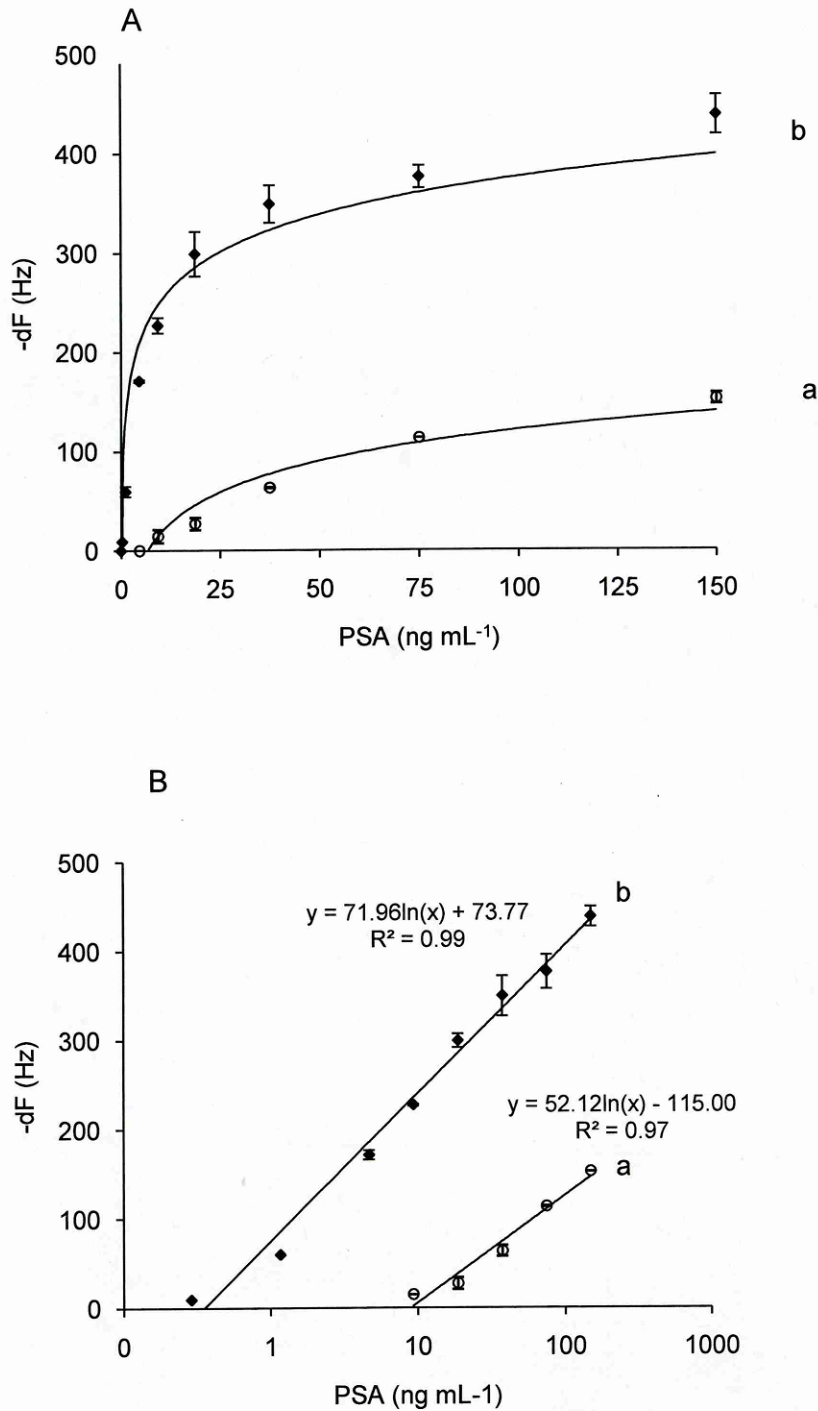


Figure 3.19. tPSA at varying concentrations (in 75% human serum) were injected to tPSA capture antibody immobilised QCM sensor surface. Later, as a sandwich assay tPSA detection antibody (a) or antibody modified Au nanoparticles (b) were injected and the frequency changes due to binding of nanoparticles were recorded and calibration curves (A - linear scale; B - log scale) were obtained.

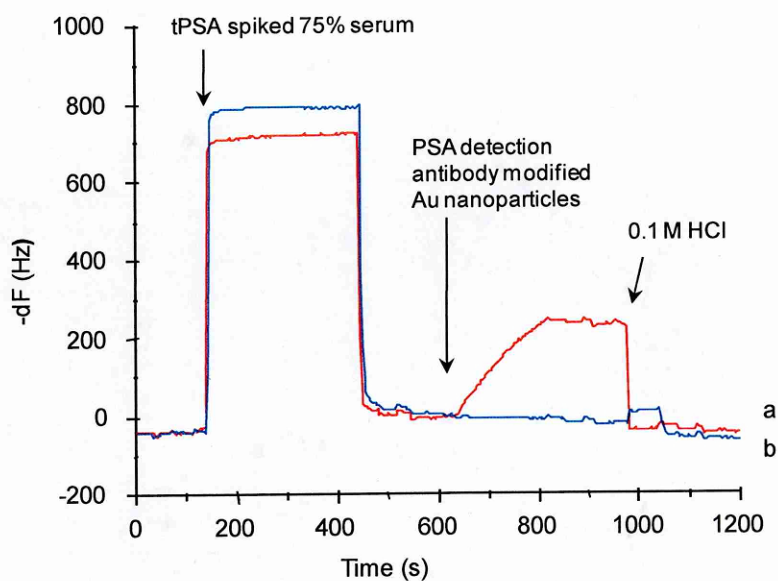


Figure 3.20. Injections of 9.4 ng ml tPSA spiked serum and tPSA antibody modified Au nanoparticle on active (a) and control (b) surfaces.

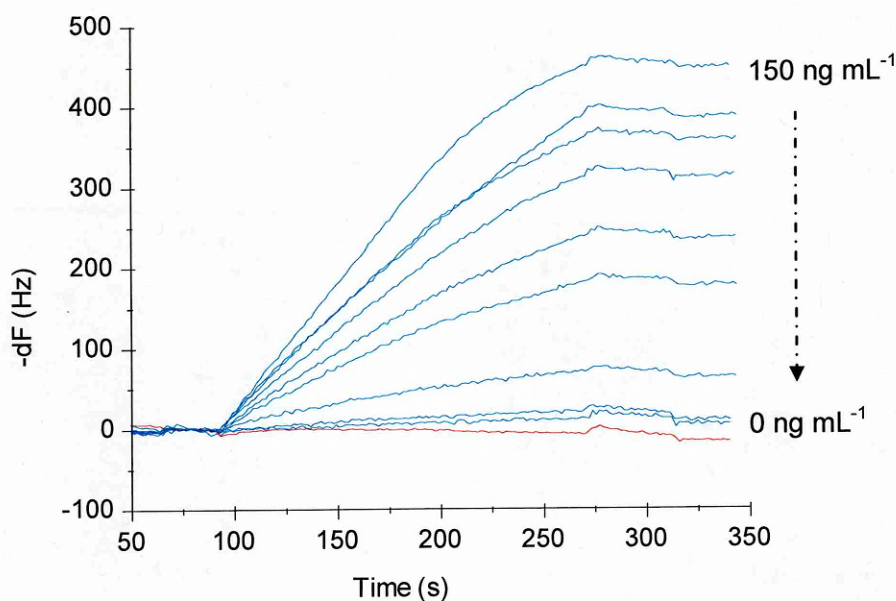


Figure 3.21. Binding of tPSA antibody modified Au nanoparticles after the injection of tPSA spiked in 75% serum at varying concentrations, from top to bottom in the order of 150, 75, 37.5, 18.8, 9.4, 4.69, 1.17, 0.29, 0 ng mL⁻¹ on tPSA antibody immobilised surface and 150 ng mL⁻¹ on MIGG immobilised surface (red).

The specificity of the tPSA interaction with the sensor surface was tested by employing a mouse IgG surface on the control sensor. The non-specific binding of PSA detection antibody modified Au nanoparticles after the injection of 75% human serum (with or without spiked tPSA) resulted in no response on mouse IgG immobilised surface (Figure 3.20- trace b). The binding of PSA detection antibody modified Au nanoparticles after the injection of 75% human serum (without spiked tPSA) resulted in 9 ± 2 Hz response on anti-PSA capture antibody immobilised surface, since non-specific binding on to MIgG surface was very low, this result was purely due to the tPSA content of the serum obtained from Sigma. While creating the calibration curve, this response was deducted from the responses obtained to get the binding results due to the spiked tPSA.

In conclusion, the limit of detection of the biomarker assay was 0.29 ng mL^{-1} (corresponds to 0.39 ng mL^{-1} in 100% patient blood serum) with a linear detection range of $0.29\text{-}150 \text{ ng mL}^{-1}$ and the coefficient of variation (CV) laid between 1 to 9% across the range. These results show that the assay developed with the formulated matrix buffer and Au nanoparticle signal amplification, have potential for the rapid and sensitive detection of serum biomarkers in patient samples. Table 3.2, summarises the results of the assays in buffer and in human serum.

Table 3.2. The summary of the PSA detection assay results.

	direct assay in PBS/T	sandwich assay in PBS/T	sandwich assay in 75% serum	sandwich assay in 75% serum using Au nanoparticles
LOD	18.8 ng mL ⁻¹	4.7 ng mL ⁻¹	9.4 ng mL ⁻¹	0.29 ng mL ⁻¹
Linear range	18.8 – 150 ng mL ⁻¹	4.7 – 150 ng mL ⁻¹	9.4 – 150 ng mL ⁻¹	0.29 – 150 ng mL ⁻¹
Equation of calibration curve	y = 0.87x + 0.27 (R ² = 1.00)*	y = 0.91x + 0.35 (R ² = 0.99)*	y = 0.89x + 0.33 (R ² = 0.98)*	y = 71.96Ln(x)+73.77 (R ² = 0.99)

*y = log (response); x = log (concentration)

The number of publications related to the detection of PSA using QCM biosensor is very limited and this indicates that application of QCM biosensors to PSA detection is a newly developing area. An example to a previous study for PSA assay using QCM biosensor has been the study performed by Zhang *et al.* which involve the use of an insert-plug model of piezoelectric immunosensor. After 40 min of reaction at 37°C in a static cell with a 10 MHz quartz crystal, Zhang *et al* achieved linear detection of 1.5 – 40 ng mL⁻¹ PSA in 100% serum (Zhang et al., 2007a). Although the detection limit obtained from this study was low enough, the reaction time was long and assay needs to be performed at elevated temperatures. Additionally the dynamic range of the assay was limited. In another study, Ding *et al.* used yeast cell modified QCM sensors to immobilise PSA antibodies (Ding et al., 2008). The binding of PSA to the PSA antibody immobilised sensor was recorded after the binding reached equilibrium using a 9 MHz quartz crystal and magnetic stirrer agitated static cell. With this method Ding *et al.* achieved detection of PSA in the range of 5 to 604 ng mL⁻¹ in 100% serum. Both applications mentioned above were performed using non-automated equipment that due to their manual operation may not only cause variations between the assays but also are not suitable for clinicians use. Additionally what attracts attention the study performed

by Ding *et al.* is that while 268 Hz frequency drop was observed from the immobilisation of anti-PSA antibody, up to 400 Hz of response was observed from the PSA binding. Using equation 3.3, it is possible to calculate the theoretical maximum response from the binding of an analyte to a ligand ($R_{\max}$ = response due to analyte binding; $MW_{\text{analyte/ligand}}$ = molecular weight of analyte [PSA, 30 000 Dalton] or ligand [anti-PSA antibody, 150 000 Dalton]; R_{ligand} = response obtained from the immobilisation of the ligand; V_{ligand} = valency of the ligand, proposed stoichiometry of the interaction) (Biacore, 2003b).

$$R_{\max} = \frac{MW_{\text{analyte}}}{MW_{\text{ligand}}} \times R_{\text{ligand}} \times V_{\text{ligand}} \quad (\text{Eq. 3.3})$$

After the immobilisation of anti-PSA antibody it is presumed to have one binding site available to bind to PSA, therefore V_{ligand} is assumed as 1 (Buhl *et al.*, 2009). In this case, for 268 Hz of anti-PSA antibody immobilisation, theoretical maximum response from PSA can be calculated as 54 Hz and even if V_{ligand} is assumed as 2, the theoretical maximum response from PSA can be calculated as 107 Hz. It is recorded in the literature that, the mass of the analyte calculated using QCM response exceeds the mass response obtained with optical techniques, and this phenomenon is explained due to the viscosity of the buffer between the bound analytes and the rigidity of the analyte layer (Hook *et al.*, 2002). However, even if this is considered, still ca. 400 Hz of response from 600 ng mL⁻¹ PSA is well above the theoretical maximum response and hence the validity of the results obtained is questionable. In the current study, (if V_{ligand} is assumed as 1) the theoretical $R_{\max}$ calculated as 76 Hz using equation 3.2 and experimental $R_{\max}$ obtained as 118 ± 11 Hz. In another study Cao *et al.* performed 10 min PSA-ACT assay in 10 % human serum using an automated SPR biosensor and obtained a detection limit

of 18.1 ng mL^{-1} that corresponds to LOD of 181 ng mL^{-1} for the whole serum (Cao et al., 2006).

In our study although 75% serum was used as assay matrix, the detection limit obtained was 0.29 ng mL^{-1} for a 8 min assay which corresponds to 0.39 ng mL^{-1} tPSA in the whole serum indicating the good performance of the optimised assay. Additionally; although high concentration of serum was used for the assay, matrix buffer limited the non-specific binding of the proteins on to sensor spots and allowed complete regeneration of the sensor surface by short injections of 100 mM HCl and 20 mM NaOH. This enables the repeated use of the same sensor chip for several times to analyse multiple serum samples to detect tPSA. The experimental conditions reported in the literature vary between the assays significantly such as, assays in static or in flow, temperature, assay time and the antibody used against PSA. All these contribute to the assay sensitivity and hence make it difficult to compare the results effectively. When compared briefly to similar label-free PSA assays such as above, the assay developed in this study, with total 8 min reaction time, repeated usability and LOD of 0.29 ng mL^{-1} in 75% human serum, proves the advantage of the optimised assay format suitable for point of care system. Additionally due to it is being rapid and wide detection range the QCM assay developed outperforms the commercial ELISA kits available for biomarker detection (Table 3.3).

Table 3.3. The summary of the PSA detection limit and detection ranges for some of the commercial ELISA kits and the developed QCM assay.

	Detection range (ng mL ⁻¹)	Limit of detection (ng mL ⁻¹)
The developed QCM assay*	0.39 – 200	0.39
Abazyme PSA ELISA kit (Abazyme, MA, USA)	1 - 80	1
MP Biomedicals PSA ELISA kit (MP Biomedicals, OH, USA)	2 - 120	1
Alpco Diagnostics PSA ELISA kit (ALPCO, NH, USA)	2 - 120	1
Calbiotech PSA ELISA kit (Calbiotech, CA, USA)	0.585 - 50	0.585
Alpha Diagnostic PSA ELISA kit (Alpha Diagnostics, TX, USA)	0.3-60	0.30

*Both detection range and limit is given based on 100% serum.

3.4.7 Testing complexed-PSA and free-PSA antibodies

In serum, PSA is found either in free form or as a complex with ACT (ACT-PSA, MW 96 kDA). Total PSA (tPSA) concentration in serum was considered normal if it is below 4 ng mL^{-1} . tPSA refers to the sum of all detectable free PSA (fPSA) and complexed PSA (cPSA) forms in the serum. The proportion of free PSA to ACT-PSA complex varies according to the type of the prostate cancer (Lilja, 2003). The commercial ELISA type assays measure total or free PSA concentration in serum. In our study we considered to incorporate either fPSA or cPSA assay next to the tPSA assay to detect tPSA and cPSA/fPSA real time simultaneously using the QCMA-1 device. For this purpose fPSA and cPSA antibodies were purchased from two companies (Alpha Diagnostics [TX, USA] and BiosPacific [CA, USA]) and their performances were investigated. The immobilised fPSA and cPSA antibodies from both manufacturers did not yield any direct binding response for their antigens and this could be due to the poor quality of the antibodies purchased. Therefore, a number of different approaches were considered to find out an assay format in which antigen binding could be observed (Table 3.4). It was found that fPSA antibodies do not bind to PSA whatever the assay method used. And cPSA antibodies (raised in mouse) from both manufacturers bind to PSA-ACT complex if captured on Rabbit anti-mouse IgG surface or used as detection antibodies for sandwich assay. The details of both assays are described in this section.

Table 3.4 Different methods used to test the performance of fPSA and cPSA antibodies using QCMA-1 sensor. The assays that did not yield any binding response were marked by ‘x’.

Tested methods	fPSA antibodies	cPSA antibodies
Immobilisation and further binding with antigen (PSA or PSA-ACT)	x	x
Dialysis of fPSA antibody, immobilisation and further binding with PSA	x	ns
Capture on RAM Fc immobilised surface and subsequent antigen binding	x	√
Capture of biotinylated cPSA antibody and subsequent antigen binding	ns	x
Immobilisation of tPSA antibody (Serotec) and sandwich assay with cPSA or fPSA antibodies	x	√
Immobilisation of tPSA antibody (Serotec) and sandwich assay with cPSA modified Au nanoparticles	ns	x
Immobilisation of biotinylated cPSA antibody (Alpha Diagnostics) and further binding with PSA	ns	x

cPSA antibody from BiosPasic (CA, USA), cPSA antibody from Alpha Diagnostics (TX, USA) and tPSA antibody from Serotec (Kidlington, UK) were captured on RAM Fc immobilised surface then binding of PSA-ACT was investigated. On the second channel cPSA antibody from Alpha Diagnostics was immobilised, to see the binding response of PSA-ACT complex to the immobilised and captured antibodies side by side. No PSA-ACT binding was observed on the immobilised cPSA antibody, whereas cPSA antibodies that were captured on RAM Fc surface did bind to PSA-ACT complex,

however the binding was much lower with respect to the tPSA antibody from Serotec (Figure 3.22).

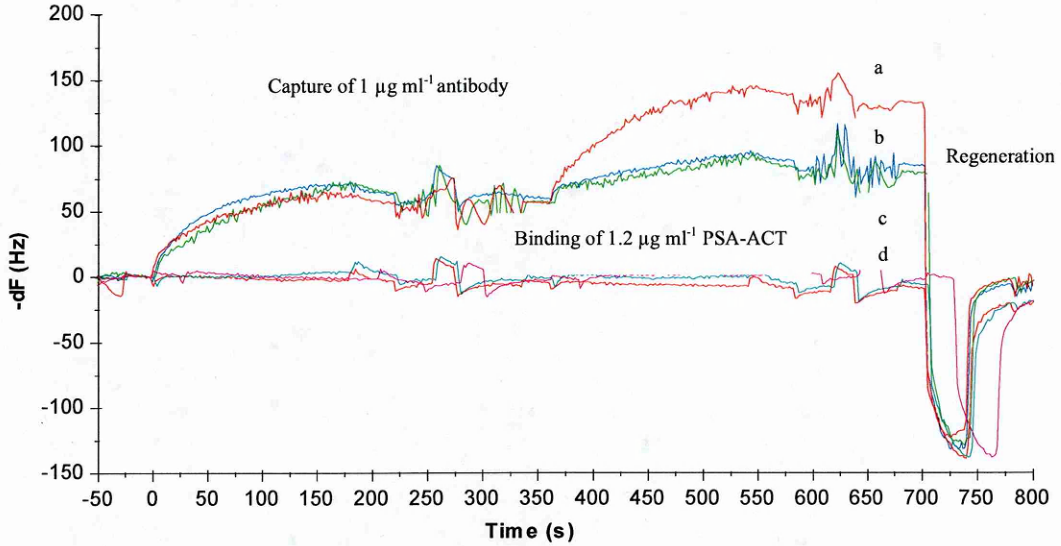


Figure 3.22. Capture of (a) tPSA antibody from Serotec, (b) cPSA antibody from Biospecific, (c) cPSA antibody from Alpha Diagnostics on RAM Fc immobilised surface and further binding of PSA-ACT complex. (d) Binding of PSA-ACT complex on cPSA antibody (Alpha Diagnostics) immobilised surface. The assay was performed using the QCM-A-1 biosensor.

Using equation 3.4, it is possible to calculate the theoretical maximum response from the binding of an analyte to a ligand ($R_{\max}$ = response due to analyte binding; $MW_{\text{analyte/ligand}}$ = molecular weight of analyte [PSA-ACT, 90 000 Dalton] or ligand [anti-PSA antibody, 150 000 Dalton]; R_{ligand} = response obtained from the capture/immobilisation of the ligand; V_{ligand} = valency of the ligand, proposed stoichiometry of the interaction).

$$R_{\max} = \frac{MW_{\text{analyte}}}{MW_{\text{ligand}}} \times R_{\text{ligand}} \times V_{\text{ligand}} \quad (\text{Eq.3.4})$$

The capture of mouse antibodies on RAM Fc immobilised surface creates an oriented layer of mouse antibodies due to the binding of mouse antibodies from the Fc regions to

the RAM Fc. This enables the use of both active sites of the antibodies for further antigen binding. The experimental results of antigen binding responses to the antibodies and the expected antigen binding responses calculated using the equation 3.6 were compared for all three antibodies. As seen from Figure 3.23, theoretical and actual PSA-ACT binding to captured tPSA antibody was very close to each other for 1-2 interaction indicating that almost all the antibodies that were captured did bind to two PSA-ACT molecules. For cPSA antibodies the actual binding was even lower than the theoretical binding results for 1-1 interaction. This clearly shows that the affinities of both cPSA antibodies are very low towards PSA-ACT if compared to tPSA antibody.

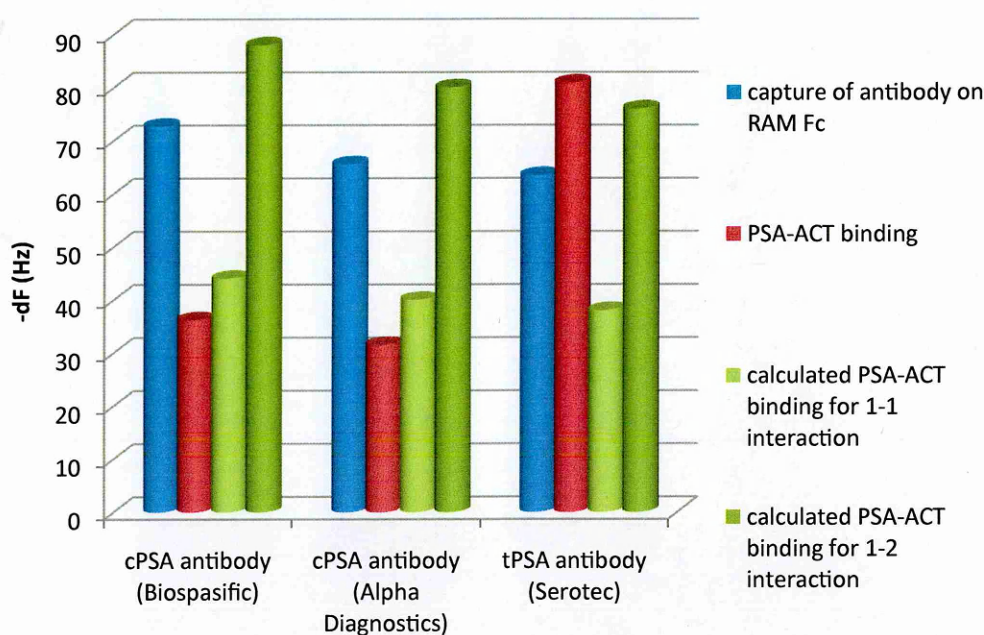


Figure 3.23. Testing the cPSA antibodies from two producers and comparison with the tPSA antibody from Serotec using QCMA-1 instrument.

As RAM Fc captured cPSA antibodies did not yield high PSA-ACT bindings, instead of using cPSA antibodies as capture antibodies, they were considered as detection antibodies for a sandwich PSA-ACT assay. For that purpose, tPSA capture antibody

(Serotec) was immobilised and after PSA-ACT capture, cPSA antibody from Biospecific was injected. Although cPSA antibody did bind to PSA-ACT complex, when compared to tPSA antibody still the affinity was low (Figure 3.24). Due to low affinity cPSA antibodies will result in high detection limit that prevents their use for clinical PSA-ACT testing.

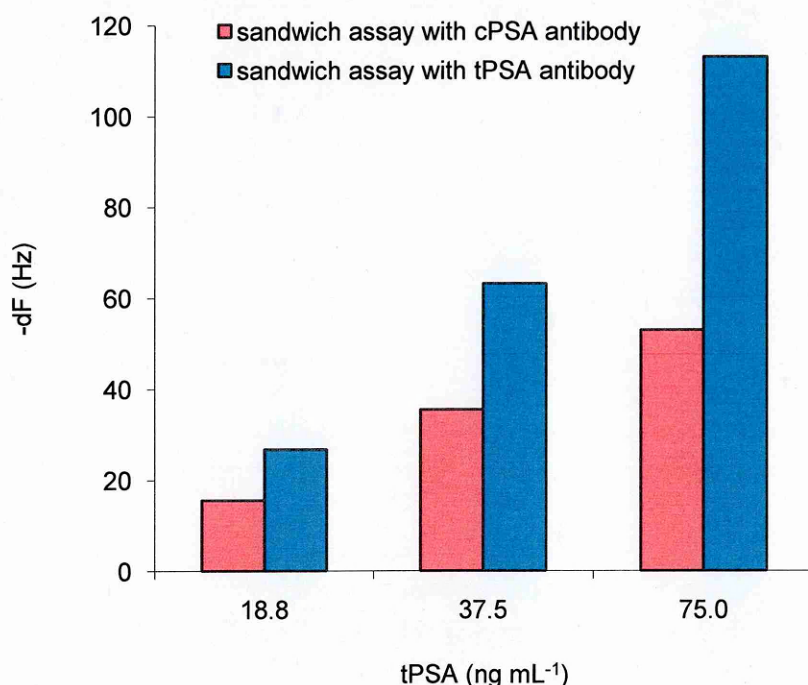


Figure 3.24. Sandwich assay using cPSA (Biospecific) and tPSA (Sertotec) antibodies after the injection of PSA spiked 75% human serum. The assay was performed using a QCMA-1 instrument.

To enhance the binding response 40 nm Au nanoparticles were modified with cPSA antibody and the assay was repeated. Unfortunately this time no binding of cPSA antibody modified Au nanoparticles were observed after PSA-ACT capture. This prevents to reach the clinically required detection limits for the PSA-ACT assay.

In our study we considered to incorporate either fPSA or cPSA assay next to the tPSA assay to detect tPSA and cPSA/fPSA real time simultaneously. For this purpose fPSA

and cPSA antibodies were purchased from two companies and their performance were investigated. It was found that fPSA antibodies do not bind to PSA whatever the assay method used. And cPSA antibodies (raised in mouse) from both manufacturers bind to PSA-ACT complex if captured on Rabbit anti-mouse IgG surface or used as detection antibodies for sandwich assay. However, the affinity of both cPSA antibodies is very low and also if the antibodies are used for Au modification they lose their activity. Hence cPSA antibodies from Alpha Diagnostics and BiosPasific were found in this study using the conditions listed to be unsuitable for complexed PSA detection.

3.4.8 Test of patient samples

Patient (from Bedford Hospital NHS Trust) and healthy volunteer (from Cranfield University staff) samples were collected for two different studies to be conducted by Emmanuel Nna and the study described in this thesis. Emmanuel Nna performed tests on patient urine and blood samples using genetic engineering tools to perform molecular profiling of prostate cancer. Although the blood samples from controls were processed as serum, the blood samples from Bedford Hospital NHS Trust were processed as plasma. Plasma is a yellowish liquid containing fluid portion of blood in which blood cells and fibrinogen are suspended. Serum is the plasma without fibrinogen or other clotting factors. As the QCMA-1 assay was optimised to perform assays to detect PSA in serum, the plasma obtained from patients' blood caused major problems while testing patient samples. Plasma and serum samples were kept at -80 °C for storage and after thawing the frozen plasma for use, some particulates were found in the solution. The QCMA-1 device has a microfluidic system and does not tolerate the use of liquids with particulates as they may block the microfluidic channel. Therefore the plasma samples were spinned for a minute at 5000 rpm and the supernatant was used for the QCMA-1 assay.

Serum or plasma obtained from patients and healthy volunteers were injected to tPSA capture antibody immobilised QCMA-1 sensor surface. Later, as a sandwich assay tPSA detection antibody modified Au nanoparticles were injected and the frequency changes due to binding of nanoparticles were recorded. Then tPSA concentrations were calculated using the calibration curves and equations from Figure 3.19 and plotted against the tPSA concentration obtained from ELISA at Bedford Hospital NHS Trust referral. As seen from the Figure 3.25, while all the control serum samples from healthy volunteers had concentrations of PSA below 2.5 ng mL⁻¹, patient samples had much

higher PSA concentrations. Although 60 samples were collected from patients only 11 of them were tested using the QCMA-1 device. The reason for that was, although plasma samples were spinned this was not enough to get rid of all the fibrinogen and it was observed that after the assay sensor surface regeneration was not possible as fibrinogen was stuck to the sensor surface. Therefore, a new sensor chip needed to be used for each sample. As there was limited amount of antibody available for the tests after the 11th sample testing was stopped. The fibrinogen not only prevented regeneration of the sensor surface but also as it blocked some of the sensor surface, reduced the sensitivity of the test. It was presumed that this led to the lower results obtained from the QCMA-1 test with respect to the PSA concentration measured at the referral to the hospital. It is important to note that there was time difference between the PSA test at referral and blood collection from the hospital for the QCMA-1 test; therefore, it was natural to see some differences in PSA concentration between the two measurements as PSA concentration may alter from day to day. Keeping these in mind, still the QCMA-1 test was able to distinguish the healthy samples from the patients as seen from Table 3.5 and Figure 3.25. Only one of the patient samples had a PSA concentration of more than 100 ng mL⁻¹, the rest of the patient samples had PSA concentrations between 4 to 20 ng mL⁻¹. Although there was problems in testing due to the obtained plasma instead of serum as described above, still PSA levels were significantly lower in the Cranfield University volunteers and below the clinical level (2.5 ng mL⁻¹) compared to patient groups in Bedford Hospital NHS Trust showing that QCMA-1 device has potential to be used for PSA testing (Figure 3.26). To get more accurate results sample handling has great importance and optimisation of sample preparation is considered as future work.

Table 3.5 The summary of the results obtained from the testing of patient and control samples using QCMA-1 sensor.

Results	Healthy controls	Patients
Average age	57± 5	63± 4
Average PSA* (measured using QCMA-1 biosensor)	0.9 ± 0.2 ng mL ⁻¹	7.8 ± 4.7 ng mL ⁻¹
Range of PSA* (measured using QCMA-1 biosensor)	0.6 to 1.2 ng mL ⁻¹	3.8 to 19.9 ng mL ⁻¹
Average PSA at referral (ELISA)	-	11.6 ± 23.9 ng mL ⁻¹
Range of PSA at referral (ELISA)	-	4.6 to 23.9 ng mL ⁻¹

*Excluding the results from patient 1 as it was a very high PSA result.

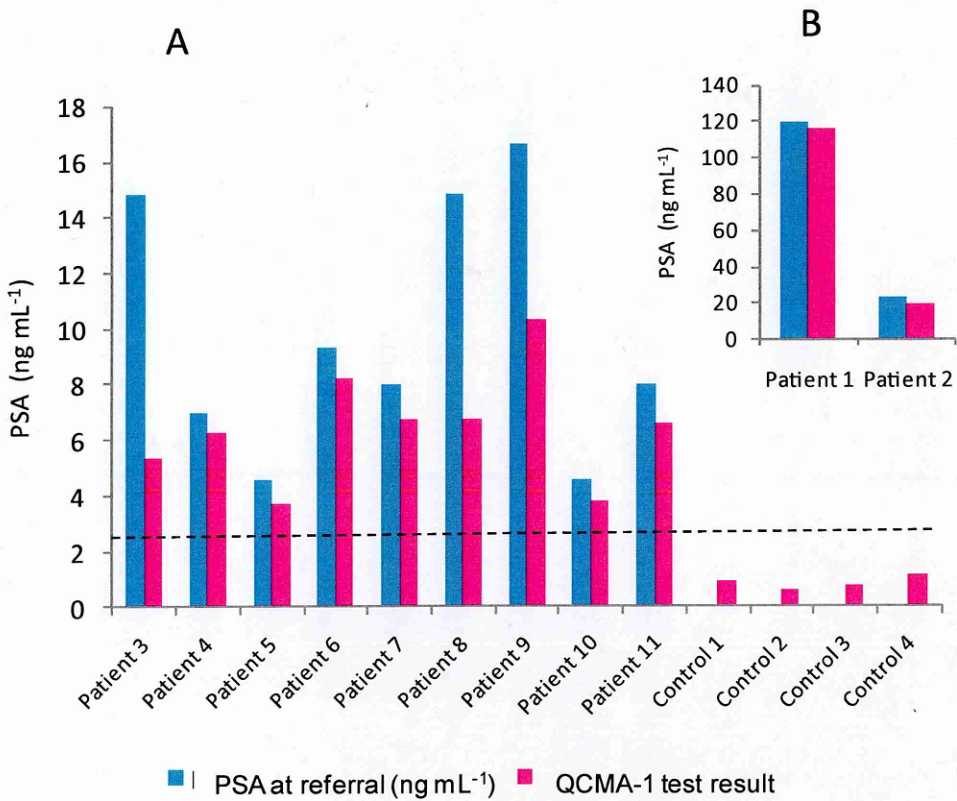


Figure 3.25. The tPSA concentration results of patient and control samples obtained using QCMA-1 instrument was plotted against the results obtained from ELISA test at referral from Bedford Hospital NHS Trust.

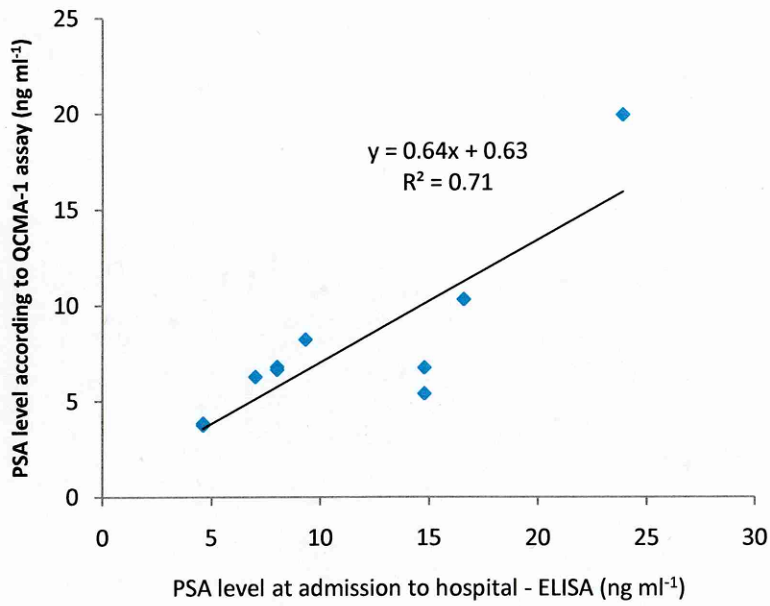


Figure 3.26. The tPSA concentration results of patient and control samples obtained using QCMA-1 instrument was plotted against the results obtained from ELISA test at referral from Bedford Hospital NHS Trust.

3.5 DEVELOPMENT OF AN SPR SENSOR FOR RESULTS AND DISCUSSION FOR BIACORE 3000 ASSAY

3.5.1 Determination of non-specific binding to the sensor surface

Bare gold Biacore sensor chips were initially coated with MUDA and PSA capture antibody and mouse IgG were immobilised using conventional EDC-NHS chemistry as reported in the methods section in this chapter. PSA capture antibody immobilised sensors produced an average response of 1247 ± 95 RU and mouse IgG immobilised sensors produced response of 2270 ± 120 RU (Figure 3.27).

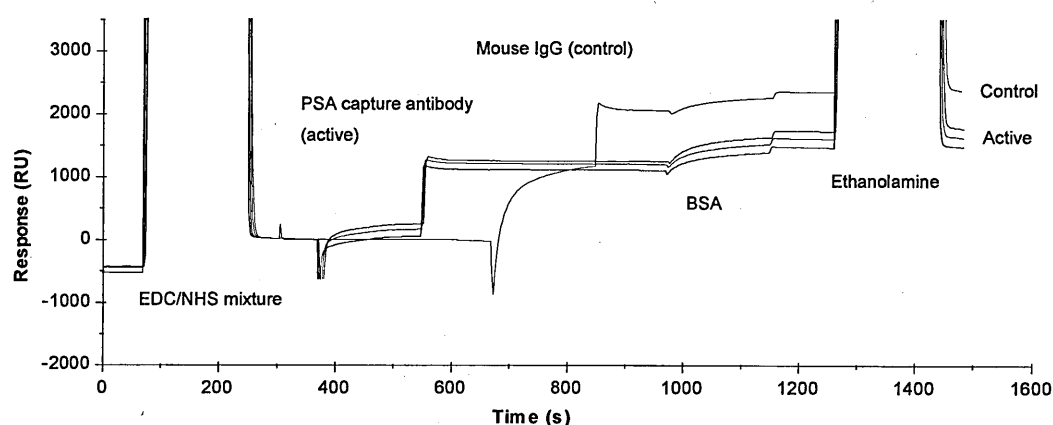


Figure 3.27. Sensorgram of PSA capture antibody (active, three flow cells) and mouse IgG (control, one flow cell) immobilisation on an MUDA coated Biacore sensor chip.

Initially the specificity of the detection was tested by performing several assays using both PSA capture antibody and mouse IgG immobilised sensor surfaces and the summary is displayed in Table 3.6. A 30 μ l 75% human serum was injected on mouse IgG immobilised sensor surface and subsequent injections of either PSA detection antibody or PSA detection antibody modified Au nanoparticles showed the degree of nonspecific binding. The non-specific binding responses were subtracted from the data to get net antigen binding results and these were presented in the following sections.

Additionally 75% human serum was injected over the PSA capture antibody immobilised surface and subsequent injection of PSA detection antibody (or its Au nanoparticle conjugates) showed the degree of response due to the existing PSA antigen in the serum. The concentration of natural PSA in the serum was lower than the detection limit of the sandwich assay therefore there was no difference in PSA detection antibody binding either it was injected directly to the PSA capture antibody immobilised sensor surface (14 ± 3 RU) or after the serum injection (13 ± 6 RU). However the detection limit of sandwich assay with PSA detection antibody modified Au nanoparticles (20 nm or 40 nm size) were lower therefore the binding of PSA detection antibody modified Au nanoparticles gave higher responses on PSA capture antibody immobilised and serum injected surfaces with respect to mouse IgG immobilised and serum injected surfaces. For example, the binding of tPSA detection antibody modified 40 nm Au nanoparticles after the injection of 75% human serum resulted in 376 ± 10 RU response on PSA capture antibody immobilised surface, very small portion of this result was non-specific binding (2 ± 6 RU) the rest was due to the tPSA content of the serum obtained from Sigma. While creating the calibration curve, this response was deducted from the responses obtained to get the binding results of the tPSA spiked serum.

Table 3.6. The results of the non-specific binding determination for all different assay formats.

Sensor surface Injected sample	Mouse IgG immobilised control surface	PSA capture antibody immobilised active surface	Mouse IgG immobilised and human serum injected control surface	PSA capture antibody immobilised and human serum injected surface
5 $\mu\text{g mL}^{-1}$ BSA	8 ± 1 RU	8 ± 6 RU	-	-
3 $\mu\text{g mL}^{-1}$ anti-PSA detection antibody	4 ± 2 RU	14 ± 3 RU	3 ± 4 RU	13 ± 6 RU
PSA detection antibody modified 20 nm Au nanoparticles	13 ± 8 RU	52 ± 8 RU	34 ± 6 RU	53 ± 7 RU*
PSA detection antibody modified 40 nm Au nanoparticles	-	-	2 ± 6 RU	376 ± 10 RU*

*The biosensor responses include not only non-specific binding but also specific binding of PSA existent in the serum used for the studies. To get the response from the spiked PSA, this response needed to be subtracted from the spiked PSA binding responses.

3.5.2 Detection of PSA in 75% human serum

One main disadvantage of label-free biosensors such as SPR is the sensitivity of the sensor to any refractive index change of the solution. If an antigen is tried to be detected from a clinical sample such as serum, refractive index mismatch of buffer and running buffer becomes problematic. While it is possible to use a standard, control serum solution that does not contain elevated levels of antigen as running buffer, it is not feasible as it is not only costly to consume that much serum for a test but also decontamination of the instrument and health and safety reasons. The only solution to this problem is to perform a sandwich assay using a detection antibody instead of a direct assay. Therefore, a sandwich assay approach was followed employing PSA detection antibody or antibody modified 20 nm Au nanoparticles. The PBS buffer containing 200 µg/mL BSA, 0.5 M salt, 500 µg/mL dextran and 0.5% Tween 20 were used to dilute human serum for the assay to reduce non-specific binding of human sera proteins to the sensor surface. The contents of this new buffer were obtained after an optimisation assay using a QCM biosensor as it is described in Section 3.4.5. As it could be seen from Figure 3.28 that when tPSA spiked 75 % human serum was injected, a high bulk shift observed around 12 000 RU due to the high refractive index of the solution. This prevents any direct tPSA binding measurements; however, subsequent injection of PSA detection antibody can be detected (Figure 3.28).

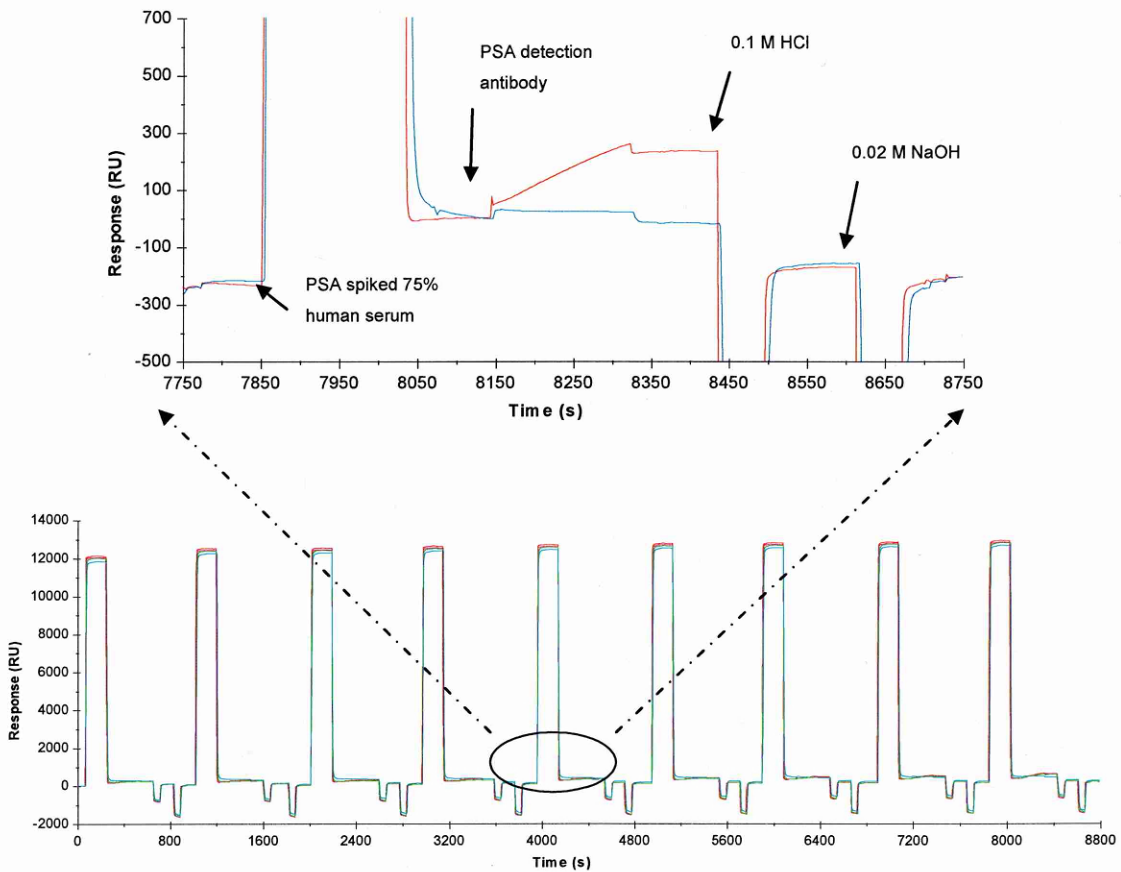


Figure 3.28. Sensorgram of the detection of tPSA spiked in 75 % human serum at varying concentrations of PSA (2.3 to 150 ng mL^{-1}). After each binding assay the surface was regenerated with consecutive 60s injections of 0.1 M HCl and 0.02 M NaOH .

The detection limit of the tPSA assay was improved to 0.5 ng mL^{-1} for the assay in buffer and 2.3 ng mL^{-1} for the assay spiked in 75 % human serum by using PSA detection antibody modified 20 nm Au nanoparticles (Figure 3.29).

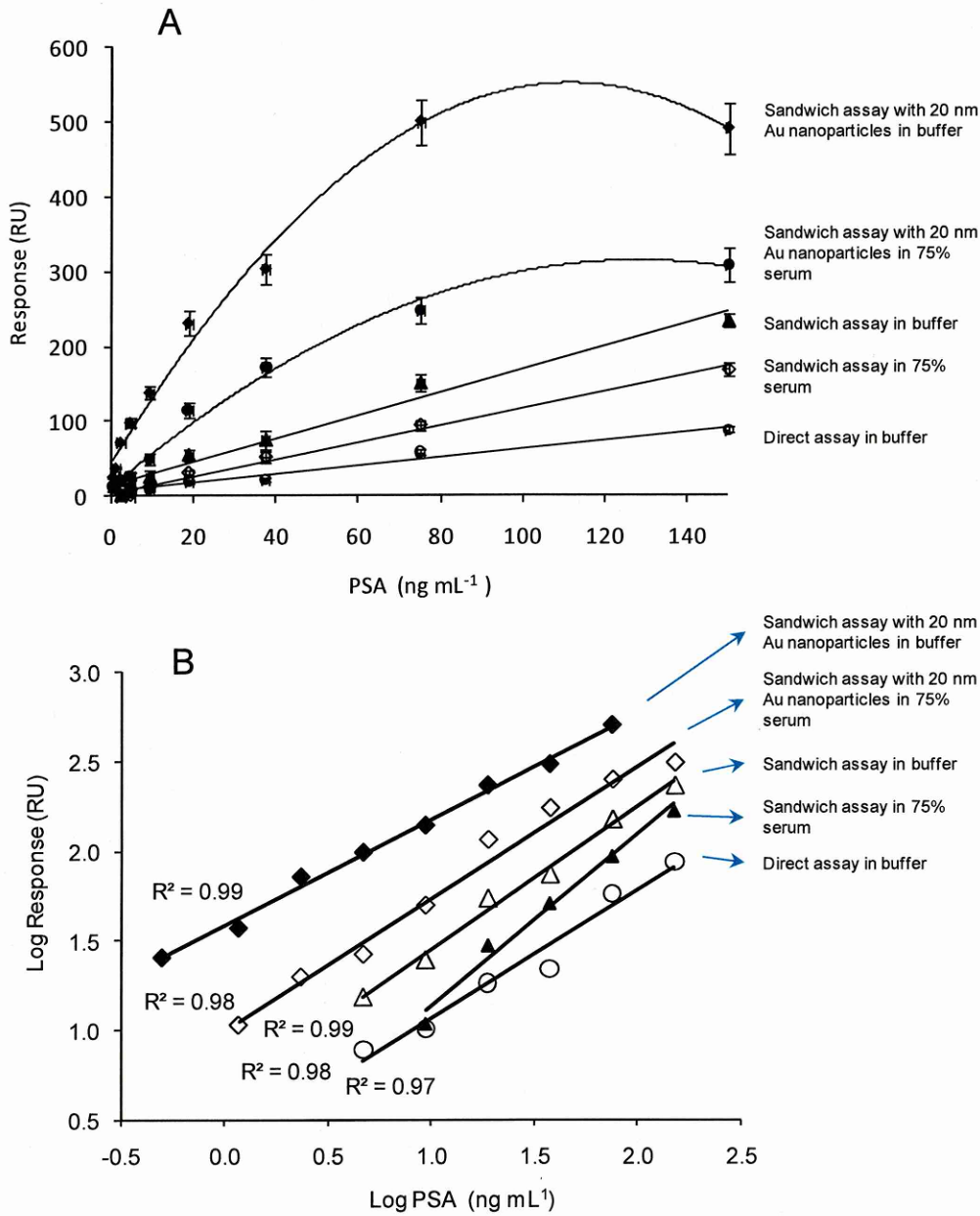


Figure 3.29. tPSA at varying concentrations (in buffer or in 75% human serum) were injected to tPSA capture antibody immobilised sensor surface. For the sandwich assay tPSA detection antibody or antibody modified Au nanoparticles were injected and the responses were recorded and calibration curves (A - linear scale; B - log scale) were obtained.

In most hospitals, the threshold PSA amount is set as 4 ng mL⁻¹ and recently there is a tendency to reduce this threshold to 2.5 ng mL⁻¹ (mainly in the USA) (Hamdan, 2007).

Patients are sent for biopsy if their total PSA level in human serum is above the threshold value (4 ng mL^{-1}). The detection limit of the PSA assay using 20 nm Au nanoparticles is low enough to detect 3.1 ng mL^{-1} in 100% human serum (equivalent of 2.3 ng mL^{-1} in 75% human serum), therefore possibility of reducing the LOD further using larger nanoparticles were considered and for that purpose the assay was repeated using 40 nm Au nanoparticles. A sample tPSA detection test using 40 nm Au nanoparticles is shown in Figure 3.30. Incorporation of 40 nm Au nanoparticles instead of 20 nm lowered the detection limit further to 0.29 ng mL^{-1} (8.5 pM) in 75% human serum (corresponds to 0.39 ng mL^{-1} in 100% serum) (Figure 3.32 and Figure 3.31). The summary of the PSA detection assay results obtained using Biacore 3000 biosensor is listed in Table 3.7.

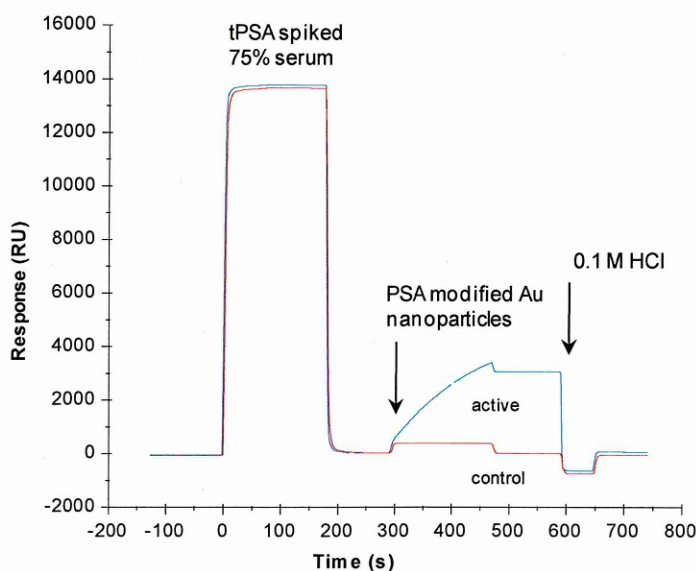


Figure 3.30. Injections of 9.4 ng mL^{-1} tPSA spiked serum and tPSA antibody modified 40 nm Au nanoparticle on active (blue) and control (red) surfaces.

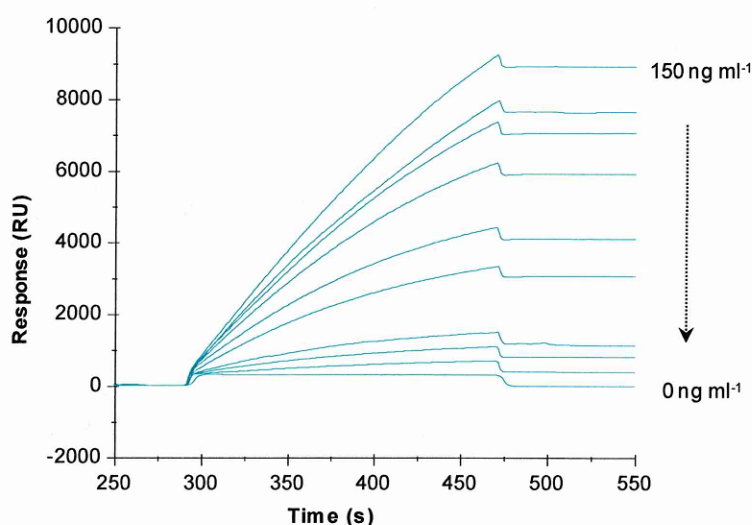


Figure 3.31. Binding of tPSA antibody modified Au nanoparticles after the injection of PSA spiked in 75% serum at varying concentrations, from top to bottom in the order of 150, 75, 37.5, 18.8, 9.4, 4.69, 1.17, 0.29, 0 ng mL⁻¹ on tPSA antibody immobilised surface and 150 ng mL⁻¹ on MIgG immobilised surface.

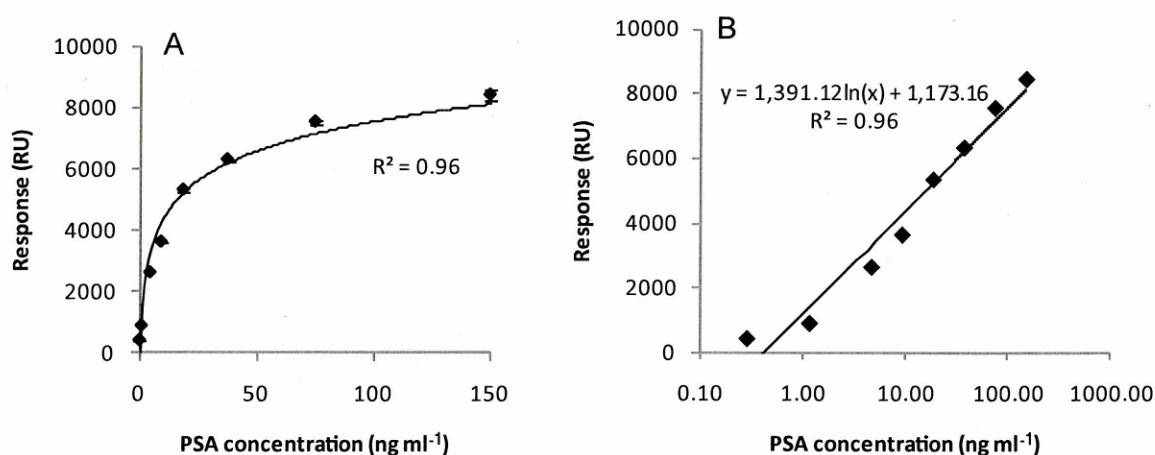


Figure 3.32. tPSA spiked in 75% human serum at varying concentrations were injected to tPSA capture antibody immobilised sensor surface. Later tPSA detection antibody modified Au nanoparticles were injected and the response changes due to binding of nanoparticles were recorded and calibration curves (A - linear scale; B - log scale) were obtained.

Table 3.7. The summary of the PSA detection assay results obtained using Biacore 3000 biosensor.

		Direct assay	Sandwich assay with PSA antibody	Sandwich assay with PSA antibody modified 20 nm Au nanoparticles	Sandwich assay with PSA antibody modified 40 nm Au nanoparticles
PSA in buffer	LOD (ng mL ⁻¹)	18.8	9.4	0.5	-
	Equation for standard curve	y = 0.72x + 0.34 (R ² = 97)*	y = 0.80x + 0.65 (R ² = 0.99)*	y = 0.59x + 1.58 (R ² = 0.99)*	-
PSA spiked in 75% human serum	LOD (ng mL ⁻¹)	-	18.8	2.3	0.29
	Equation for standard curve	-	y = 1.04x + 0.03 (R ² = 0.98)* CV = 5 to 14 %	y = 0.73x + 1.00 (R ² = 0.99)* CV = 7 to 22 %	y = 1,391.12ln(x) + 1,173.16 (R ² = 0.96) CV = 1 to 8 %

* y = log (response) x = log (concentration)

Although label-free biosensors has several advantages such as ability of real-time, continuous measurements and no need of a label for sensing; they do have two main disadvantages as limited sensitivity and the requirement of assay optimisation and proper use of controls to differentiate non-specific binding from specific especially while using complex matrices. In the current study by means of a matrix elimination buffer the non-specific sera protein binding to the sensor surface was minimised and relative control experiments were performed to prove the specificity of the bindings. However, to enhance the sensitivity to obtain clinically relevant results there was a need to use Au nanoparticle labels that compromises the label-free sensing of the detection method. Au nanoparticles cause high resonance angle shift due to their participation to surface plasmon resonance and hence increases the signal obtained from the sensor. Therefore, the nanoparticle size has an important factor on the degree of signal enhancement. In the study, while use of 20 nm Au nanoparticles reduced the detection limit 8 times, the use of 40 nm Au nanoparticles lowered the detection limit 65 times with respect to the sandwich assay with detection antibody. The signal enhancement

between 20 nm and 40 nm nanoparticles is 8 times that correlates very well with the 8 times difference in their volume.

Since the detection limit of the tPSA assay is low enough to detect 0.29 ng mL^{-1} (8.5 pM) in 75% human serum (corresponds to 0.39 ng mL^{-1} in 100% serum), it is possible to perform tPSA assay using Biacore instrument to determine the total PSA level in patient's serum.

Huang *et al.* performed PSA assay in 50% human serum and to reduce the non-specific binding of serum proteins they tested different percentages of thiols to form a mixed SAM (16-mercapto-1-hexadecanoic acid and 1mM 11-mercapto-1-undecanol) (Huang *et al.*, 2005). They found the optimum mixed SAM concentration as 10% 16-mercapto-1-hexadecanoic acid to obtain non-specific binding of ca. 500 RU for 50% human serum. They only preferred to state that using the mixed SAM it was possible to detect PSA binding if spiked in 50% human serum, however they did not perform further assays to determine LOD. As a further assay Huang *et al.* used streptavidin modified gold nanoparticles to enhance the signal and obtained 1 ng mL^{-1} detection limit for PSA assay in buffer (Huang *et al.*, 2005). In another study, Cao *et al.* performed a sandwich assay for detection of PSA-ACT complex using Biacore 2000 and the detection limit they obtained for PSA-ACT spiked in 10% human serum was 18.1 ng mL^{-1} (Cao *et al.*, 2006). Another study performed by Besseling *et al.* consists of two step signal enhancement process including sandwich assay with rabbit anti-PSA antibodies and further binding of goat anti-rabbit IgG modified Au nanoparticles (10nm) (Besselink *et al.*, 2004). A 30 minutes static assay using IBIS SPR biosensor (IBIS Technologies, Hengelo, Netherlands) resulted in LOD of 0.15 ng mL^{-1} for PSA assay in buffer. The experimental conditions vary between the assays significantly such as, assays in static or in flow, temperature, assay time and the antibody used against PSA and SPR

instrument used (Table 3.8). All these contribute to the assay sensitivity and hence make it difficult to compare the results effectively. If we compare the current study with published data briefly, as stated above after 6 minutes total reaction time, we obtained LOD of 0.29 ng mL^{-1} in 75% human serum; indicating that our optimised PSA assay outperforms the assays performed by other researchers using SPR instruments.

Table 3.8 Examples of PSA assay in the literature performed using SPR biosensors.

Analyte	Sensor surface format and/or amplification method	LOD for direct assay in buffer	LOD in buffer	LOD in serum	Assay reaction time	References
PSA& PSA-ACT	40 nm Au nanoparticle amplification, planar surface	-	-	0.29 ng mL ⁻¹ 75% serum	6 min	Uludag & Tothill
PSA	20 nm Au nanoparticle amplification, planar surface	18.8 ng mL ⁻¹	0.5 ng mL ⁻¹	2.3 ng mL ⁻¹ 75% serum	6 min	Uludag & Tothill
PSA-ACT	planar surface	20.7 ng mL ⁻¹	10.2 ng mL ⁻¹	18.1 ng mL ⁻¹ 10% serum	10 min	(Cao et al., 2006)
PSA-ACT	HRP-labelled polyclonal antibody-functionalized ca. 20nm Au nanoparticle amplification, planar surface	56.9 ng mL ⁻¹	0.027 ng mL ⁻¹	-	10 min	(Cao and Sim, 2007)
PSA& PSA-ACT	planar surface	-	3.5 ng mL ⁻¹	-	12 min	(Saerens et al., 2005)
PSA& PSA-ACT	CM5 surface	-	0.19 ng mL ⁻¹	-	12 min	(Saerens et al., 2005)
PSA	20nm Au nanoparticle amplification, planar surface	10 ng mL ⁻¹	1 ng mL ⁻¹	-	19 min	(Huang et al., 2005)
PSA	10nm Au nanoparticle amplification, planar surface	60 ng mL ⁻¹	0.15 ng mL ⁻¹	-	30 min	(Besselink et al., 2004)
PSA	20nm Au nanoparticle amplification, planar surface	-	0.01 ng mL ⁻¹	-	8 hrs	(Choi et al., 2008)

3.6 BIACORE 3000 AND QCMA-1 ASSAY COMPARISON

QCMA-1 instrument is a new prototype instrument developed by Sierra Sensors GmbH. To validate the PSA assay results obtained using QCMA-1 biosensor, a comparative study with an established commercial biosensor was considered and Biacore 3000 instrument was chosen for that purpose.

Initially, the interaction kinetic constants for PSA and PSA capture antibody were calculated using the data created from both SPR and QCM biosensors and results were compared. The interaction between an immobilised ligand and an analyte (Figure 3.33 and equation 3.5) can be described in equation 3.6, and this equation can be transformed into equation 3.7, if it is written in terms of biosensor responses [R : sensor response (RU or Hz), C : concentration of analyte (M), k_a : association rate constant, k_d : dissociation rate constant].

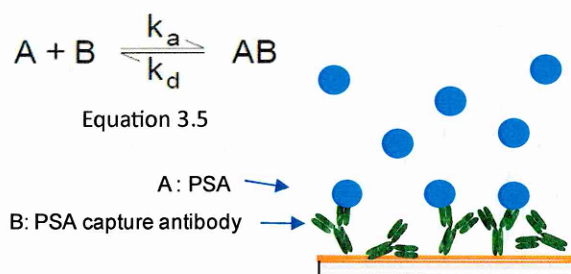


Figure 3.33. The figure shows the binding of an analyte to an immobilised ligand on the sensor surface. After the immobilisation of antibody it is presumed to have one binding site available to bind; therefore, 1:1 interaction model where one molecule (A), reversibly binds to another (B) can be used for kinetics calculations (equation 3.5).

$$\frac{d[AB]}{dt} = k_a \cdot [A] \cdot [B] - k_d \cdot [AB] \quad \text{Equation 3.6}$$

$$\frac{d[R]}{dt} = k_a \cdot C \cdot [R_{\max} - R] - k_d \cdot R \quad \text{Equation 3.7}$$

In the study, kinetic calculations were performed using BIAevaluation software (version 4.1, GE Healthcare) that uses the equations above and enables to fit the data to interaction models. The PSA binding to the PSA capture antibody immobilised surface was tested in a concentration range between 4.7 to 150 ng mL⁻¹ and PSA binding response curves were then fitted to 1:1 Langmuir binding model to determine the binding association and dissociation rates (Rich and Myszk, 2009), from which K_D value was calculated as 9.46 × 10⁻¹⁰ M using SPR instrument and 5.56 × 10⁻¹⁰ M using the QCM sensor (Table 3.9). These two results are close to each other and prove that the affinity between PSA and PSA-capture antibody is in the region of 10⁻¹⁰ M. Comparing these results with those achieved by other authors indicate good sensitivity of the assay with the used antibody. Karlsson *et al.* calculated the affinity of anti-PSA antibody towards PSA as 3.3 × 10⁻⁹ M (antibody from Fitzgerald Industries Int., clone M212091) (Karlsson *et al.*, 2006). Katsamba *et al.* calculated the affinity as 1.1 × 10⁻⁹ M (antibody from Fitzgerald Industries Int., clone M612166) (Katsamba *et al.*, 2006).

Table 3.9. Results of kinetic calculations for PSA direct assay and detection limits using SPR and QCM biosensors.

	SPR	QCM
K_D	$9.46 \times 10^{-10} \text{ M}$	$5.56 \times 10^{-10} \text{ M}$
k_a	$2.08 \times 10^{+06} \text{ M}^{-1} \text{ s}^{-1}$	$1.23 \times 10^{+06} \text{ M}^{-1} \text{ s}^{-1}$
k_d	$1.97 \times 10^{-03} \text{ s}^{-1}$	$6.83 \times 10^{-04} \text{ s}^{-1}$
R_{max}	110 RU	108 Hz

The activity of the QCM and SPR sensor surfaces were evaluated by comparing the theoretical and experimental maximum response obtained from the PSA binding to immobilised anti-PSA antibody. Using equation 3.8, the theoretical maximum response can be calculated for the binding of an analyte to a ligand (R_{max} = response due to analyte binding; $MW_{\text{analyte/ligand}}$ = molecular weight of analyte [PSA, 33 000 Dalton] or ligand [PSA antibody, 150 000 Dalton]; R_{ligand} = response obtained from the immobilisation of the ligand; V_{ligand} = valency of the ligand, proposed stoichiometry of the interaction) (Biacore, 2003b).

$$R_{\text{max}} = \frac{MW_{\text{analyte}}}{MW_{\text{ligand}}} \times R_{\text{ligand}} \times V_{\text{ligand}}$$

Equation 3.8

After the immobilisation of PSA antibody it is expected to have one binding site available to bind to PSA, therefore V_{ligand} is assumed as 1. For the assay performed using SPR biosensor, the theoretical R_{max} calculated as 274 RU using equation 1 and experimental R_{max} obtained as 110 RU, indicating 40% activity of the antibody immobilised surface. For the assay performed using QCM biosensor, the theoretical R_{max} calculated as 84 Hz using equation 1 and experimental R_{max} obtained as 108 Hz

that corresponds to 129% activity. It is recorded in the literature that, the mass of the analyte calculated using QCM response exceeds the mass response obtained with optical techniques, and this phenomenon is explained due to the viscosity of the buffer between the bound analytes and the rigidity of the analyte layer (Hook et al., 2002).

To compare the assay results of both QCM and SPR assay results further, the direct and sandwich PSA assay results in buffer using both instruments were plotted against each other. Direct and sandwich assay results obtained from SPR and QCM instrument produced a linear lines with R^2 values 0.98 and 0.99 respectively (Figure 3.34 - A), additionally sandwich assay in 75% human serum using 40 nm Au nanoparticles gave R^2 value of 0.98 (Figure 3.34 - B). This outcome indicates that the assay results of both instruments are in line with each other and both instruments can be used to detect clinical samples in high serum concentrations by performing a sandwich assay to get away from interferences from the complex media either as refractive index or viscosity/density changes. The comparison of the results obtained using both QCM and SPR biosensors can be found from Table 3.10.

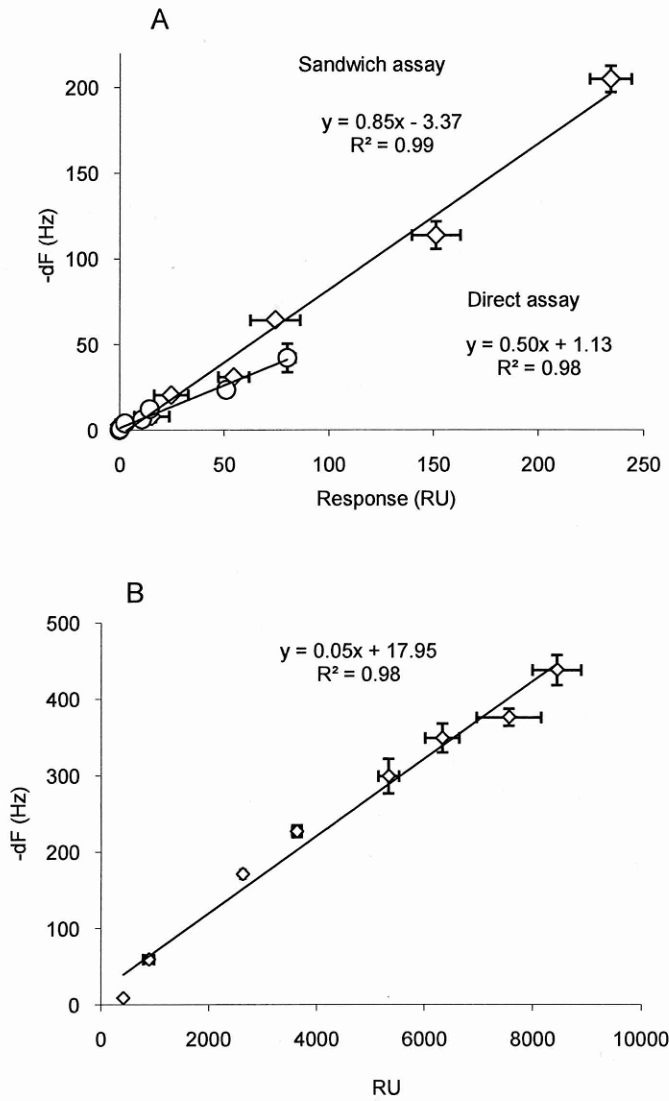


Figure 3.34. The comparison of Biacore 3000 (x axis) and QCMA-1 (y axis) responses for (A) PSA direct and sandwich assays in buffer and (B) sandwich assay using 40 nm Au nanoparticles in 75% human serum. Each data point corresponds to responses obtained from the biosensors using a different PSA concentration (2.9 – 150 ng mL⁻¹).

Table 3.10. The summary of the PSA assay results obtained using Biacore 3000 and QCMA-1 instruments.

	direct assay		sandwich assay with anti-PSA antibody		sandwich assay with anti-PSA antibody modified 40nm Au nanoparticles	
	Biacore	QCMA-1	Biacore	QCMA-1	Biacore	QCMA-1
PSA in buffer	LOD (ng mL ⁻¹)	18.8	18.8	4.7	-	-
	Equation y = log (response) x = log (concentration)	y = 0.72x + 0.34 (R ² = 97)	y = 1.10x + 0.69 (R ² = 0.94)	y = 0.80x + 0.65 (R ² = 0.99)	y = 0.91x + 0.35 (R ² = 0.99)	-
PSA spiked in 75% Human serum	LOD (ng mL ⁻¹)	-	18.8	18.8	0.29	0.29
	Equation y = log (response) x = log (concentration)	-	y = 1.04x + 0.03 (R ² = 0.96)	y = 0.67x + 0.34 (R ² = 0.99)	y = 1391Ln(x)+1173 (R ² = 0.96)	y = 71.96Ln(x)+73.77 (R ² = 0.99)

3.7 CONCLUSIONS

In this chapter, a preliminary study was described for the use of QCMA-1 biosensor to perform the PSA detection assay and then the results obtained were validated and compared using a commercial biosensor (Biacore 3000).

There is a significant difference in the limit of detection when buffer or clinical sample (serum) was used as the assay media. The change in the detection limit, when clinical samples are used, is mainly due to the lower signal to noise ratio resulting from the high non-specific binding of sera proteins/antibodies to the sensor surface. This is especially noticeable when label-free biosensors are employed, and this prevents researchers from the use of high concentrations of human serum. Therefore, most analyses are conducted using concentration of human serum in the range 10 to 50 % (Cheng et al., 2008; Kurosawa et al., 2004; Situ et al., 2008). In the current study we investigated the use of additives in the buffer to minimize the adsorption of sera proteins to the sensor surface. For that purpose a new buffer was formulated to eliminate 98% of the non-specific human serum protein binding to the sensor surface. Using this new formulated 'matrix buffer', the detection of PSA concentrations down to 0.29 ng mL^{-1} in 75% serum was achieved with a linear detection range of $0.29 - 150 \text{ ng mL}^{-1}$ using both instruments with Au nanoparticles employed for sensitivity enhancement. Both QCMA-1 and Biacore 3000 PSA binding data fitted to 1:1 Langmuir binding model and K_D calculated in the region of 10^{-10} M for both. The achieved results indicate that data obtained from both QCMA-1 (Sierra Sensors GmbH , Germany) and Biacore 3000 (GE Healthcare, Sweden) instruments are compatible with each other and additionally both instruments can be used for the fast and easy detection of PSA in patient samples.

In the study incorporation of fPSA, cPSA and PSMA immunoassays to the prostate cancer detection test was considered. For this purpose fPSA and cPSA antibodies were purchased from two companies and their performances were investigated. PSMA and fPSA antibodies did not work for the assay at all and the affinity of cPSA antibodies were very low and not suitable for the assay. This result very clearly shows that the performance of the biosensor assays depend on the performance of the antibodies used for the immunoassay.

CHAPTER IV:
CAPACITIVE BIOSENSOR DEVELOPMENT FOR PSA
DETECTION

4 CAPACITIVE BIOSENSOR DEVELOPMENT FOR PSA DETECTION

In the study two label-free biosensors, piezoelectric (QCM) and optical (SPR) were applied for the detection of PSA. As an addition to these, a label-free electrochemical sensor in the form of capacitive sensing was also investigated. Capacitive biosensors have potential to be miniaturised and turned into a hand held device for point-of-care sensing. A collaboration study was performed with Sabanci University (Istanbul, Turkey) where capacitive sensor chips were fabricated and tested. Capacitive biosensors chips do consist of interdigitated electrode (IDE) arrays and were fabricated with conventional microelectronics-micromachining technologies on silicon wafers in a class 1000 clean room. Later IDE arrays were utilised for the detection of PSA using a network analyzer or an LCR meter.

4.1 MATERIALS AND INSTRUMENTATION

Mercaptoundecanoic acid (MUDA), spectrophotometric grade ethanol, potassium ferricyanide, phosphate buffered saline (PBS, 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4), bovine serum albumin (BSA), ethanolamine, N-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) were purchased from Sigma-Aldrich (Interlab A.S., Istanbul, Turkey). Potassium chloride (KCl) and tetraethoxy silane was purchased from Fisher Scientific. PSA, anti-PSA detection and capture antibodies were bought from AbD Serotec, (Kidlington, UK). Ultrapure water ($18\text{ M}\Omega\text{ cm}^{-1}$) was obtained from a Milli-Q water system (Millipore Corp., Tokyo, Japan).

For capacitance measurements Agilent-8720ES S-Parameter Network Analyzer (CA, US) (Figure 4.1 - A), Karl-Suss PM-5 RF Probe Station (Figure 4.1 - B) and 41R LCR meter from CHY FirematenC (Taiwan) were used. For cyclic voltammetry BAS-Epsilon potentiostat was employed. Gold coated interdigitated electrode arrays (IDA) (Figure 4.1 - C), were fabricated on silicon wafer or glass slide using lithography techniques in a class 1000 clean room. Length of each electrode is 750 microns and the width and the distance between the electrodes is 25 microns each.

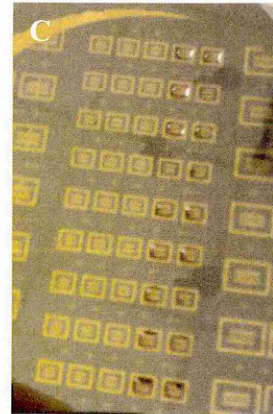
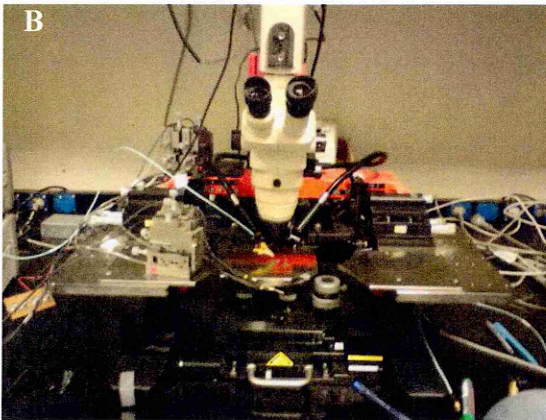
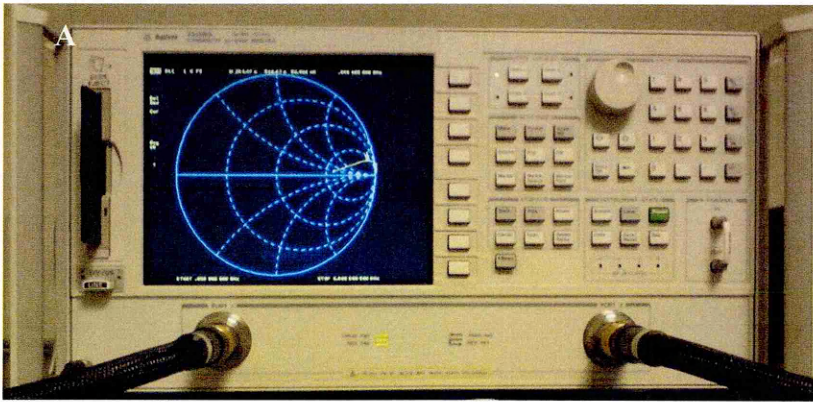


Figure 4.1. (A) Agilent-8720ES S-Parameter Network Analyzer. (B) Karl-Suss PM-5 RF Probe Station (C) Interdigitated electrode arrays on a silicon wafer.

4.2 METHODS

4.2.1 Fabrication of IDEs

For the design of interdigitated electrodes (IDE), two arrays of electrodes with comb shape were utilised. Interdigitated electrode arrays were fabricated with using conventional microfabrication technologies on standard glass slides and silicon wafers in a class 1000 clean room (Sabanci University, Turkey). Initially, the substrates were cleaned in a piranha solution (sulphuric acid: hydrogen peroxide, 3:1) and rinsed with copious amount of DI water. Then lithography techniques were applied to create the image of the electrodes on the substrate by photoresist coating and UV exposure using a template. The substrate was sputtered with Ti (~30nm) and Au (~200nm) which was followed with a lift off process to create the interdigitated electrodes (Figure 4.2).

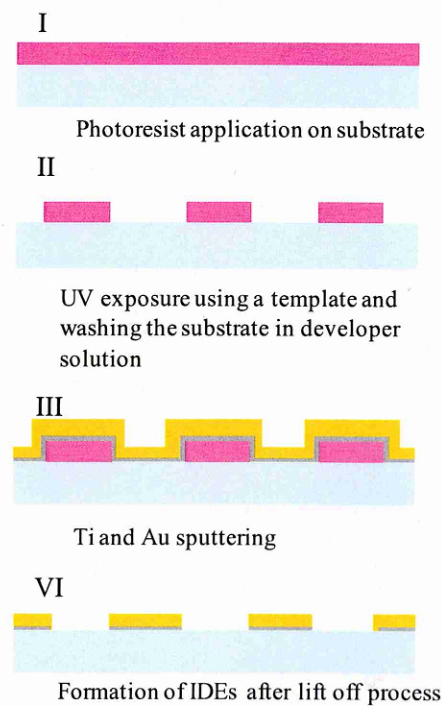


Figure 4.2. Schematics of IDE fabrication on substrates.

4.2.2 Surface chemistry application

The silicon wafer/glass slide was plasma cleaned with argon plasma for two minutes using a plasma etcher. The silicon wafer/glass slide was then transferred to the SAM deposition Petri dish filled with 2 mM MUDA or 2 mM epoxy silane solution. The Petri dish was covered with aluminium foil and incubated overnight at ambient temperature. Next morning the silicon wafer/glass slide was rinsed with ethanol and water and dried with nitrogen stream. The SAM coated sensors were placed in a Petri dish and stored in the fridge till further use. If further blocking of the silicon surface was required, the SAM coated silicon wafer was incubated with a silane solution for 2 hours at ambient temperature. After rinsing the silicon wafer/glass slide with methanol and deionised water, then it is dried with nitrogen stream and stored in the fridge for further use.

4.2.3 Sensor surface preparation and antigen binding

Sensor surfaces were prepared by immobilising mouse anti-PSA capture antibody on interdigitated electrodes (IDE) that were coated with MUDA or epoxysilane. The immobilisation was achieved using conventional amine coupling chemistry for MUDA coated sensors. Sensor surfaces were first activated with 2 μ l 1 : 1 mixture of 400 mM EDC and 100 mM NHS, prepared in 0.22 μ m filtered deionised water, and mixed immediately prior to use (final concentrations: 200 mM EDC and 50 mM NHS) for 5 minutes. Then 2 μ l, 30 μ g mL⁻¹ PSA capture antibody (in sodium acetate buffer, pH 5.5) was placed on electrodes and incubated for 60 minutes at room temperature. The surface then blocked with 2 μ l, 50 μ g mL⁻¹ BSA in PBS for 30 minutes. Non-reacted NHS esters were capped with 1 M ethanolamine, pH 8.5 for 5 minutes. Between each process the IDEs were washed with PBS (pH 7.4) and

deionised water, then dried with a nitrogen stream. The capacitance measurements were taken before and after the immobilisation using network analyzer and probe station.

After antibody immobilisation process the sensor surface was ready for antigen binding experiments. PSA was diluted at specified concentrations in PBS buffer containing $5 \mu\text{g mL}^{-1}$ BSA. A $2 \mu\text{l}$ PSA at specified concentrations was placed over PSA detection antibody and anti-CRP immobilised surfaces for 30 minutes to allow binding. The IDEs were washed with PBS and deionised water and dried using a nitrogen stream. The capacitance measurements were taken after antigen binding using the network analyzer and probe station. A $2 \mu\text{l}$ PSA detection antibody ($1.5 \mu\text{g mL}^{-1}$) was then placed on the PSA bound surfaces to perform a sandwich assay (30 min) (**Figure 4.3**). After drying the IDEs, the capacitance measurements were taken.

For the continuous assay measurements in buffer, the capacitor was dipped in an Eppendorf containing $400 \mu\text{l}$ buffer and the assay was performed by changing the buffer with assay reagents as mentioned above. And the capacitance measurements were taken real-time using an LCR meter connected to a computer.

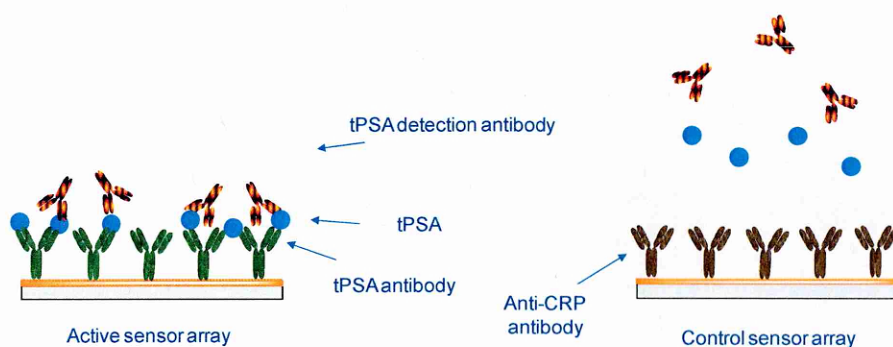


Figure 4.3. The schematic of PSA detection assay.

4.2.4 Capacitance measurements using network analyzer and probe station

The experiments were performed using an Agilent-8720ES S-Parameter Network Analyzer and Karl-Suss PM-5 RF Probe Station. Network Analyzer was calibrated using SOLT (short-open-load-through) method and S-Parameter data of the capacitor were measured. The frequency range tested was between 0.05 GHz to 6 GHz using the probe station (Figure 4.4) and the network analyzer. After the measurements taken the capacitance of the IDA was calculated using equation 4.1 (X_c = capacitive reactance in ohms, (Ω); f = frequency in Hertz, (Hz); C = capacitance in Farads, (F); π = (pi) = 3.142). To ease the complicated calculation process, an excel template was created that automatically recalled the data from the folders and calculated the capacitance for the frequency range (0.05 – 6 GHz).

$$C = \frac{1}{2\pi X_c f} \quad \text{Equation 4.1}$$

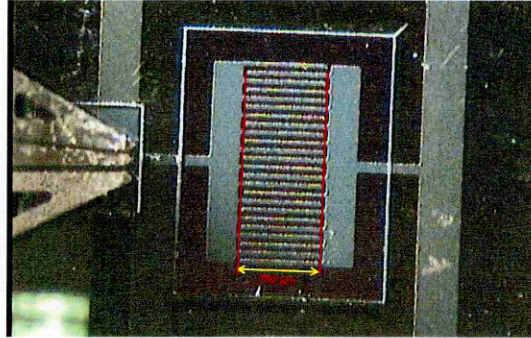


Figure 4.4. Measurement of the capacitance of an interdigitated electrode using probe station.

4.3 RESULTS AND DISCUSSION

4.3.1 Capacitive biosensor development using network analyzer and probe station

Agilent Network Analyzer and Karl Suss probe station instruments were employed as capacitive detection platform for PSA detection. In the current study interdigitated electrodes (IDE) consist of two arrays of electrodes with comb shape were utilised. Interdigitated electrode arrays were fabricated with conventional microelectronics-micromachining technologies on silicon oxide wafers in a class 1000 clean room. Interdigitated electrodes with three surface chemistries were fabricated and tested to compare their assay performances:

- a. Wafer A: Silicon oxide surface between the Au electrodes was modified with epoxy silane for antibody immobilisation and Au coated electrodes were left blank
- b. Wafer B: Au coated electrodes were modified with MUDA for antibody immobilisation
- c. Wafer C: Au coated electrodes were modified with MUDA for antibody immobilisation and silicon oxide area between the electrodes was blocked with a blocking silane (tetra-ethoxy silane)

Capacitances of the IDEs were measured before and after surface modification at the frequency range 0.05 – 6 GHz (Figure 4.5). The capacitance change due to surface modification varies between different capacitors as seen in Figure 4.6.

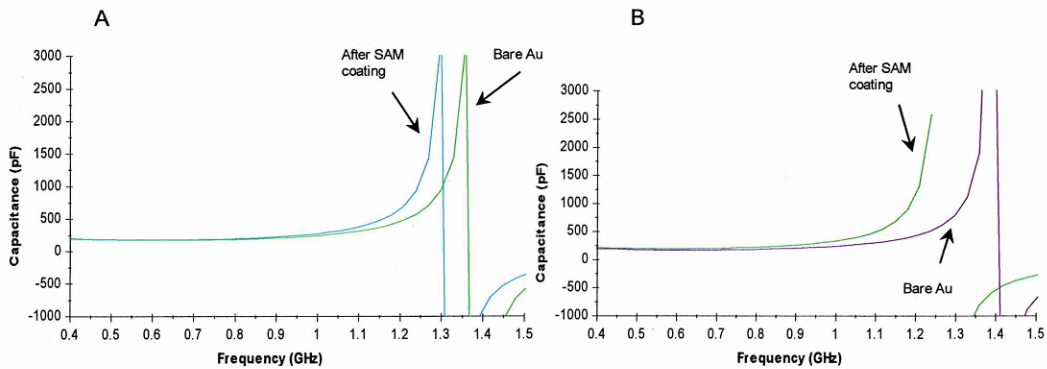


Figure 4.5. Capacitance measurement of two IDEs number 1 (A) and 2 (B) before and after SAM coating. Measurement taken using a network analyzer and a probe station.

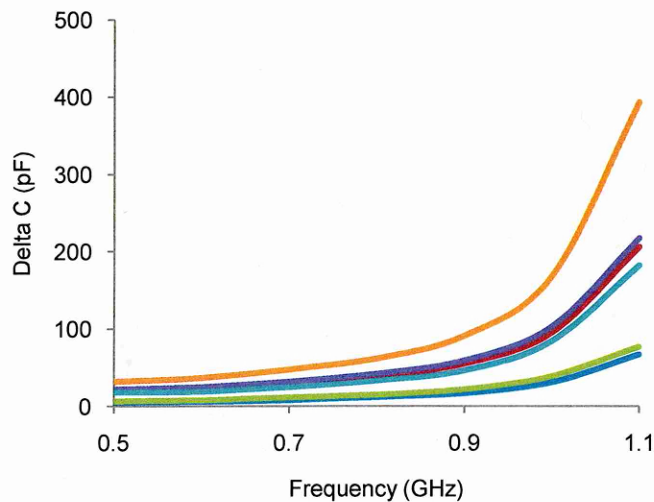


Figure 4.6. Capacitance measurements of 6 IDEs were measured before and after SAM coating and the results were subtracted from each other to obtain the capacitance change due to surface modification. There is large variation in the capacitance change between different sensors.

The capacitance change due to PSA capture antibody immobilisation was in the range of 40 pF apart from two capacitors, which showed 100 and 180 pF capacitance change (all at frequency 1GHz) (Figure 4.7 and Figure 4.8).

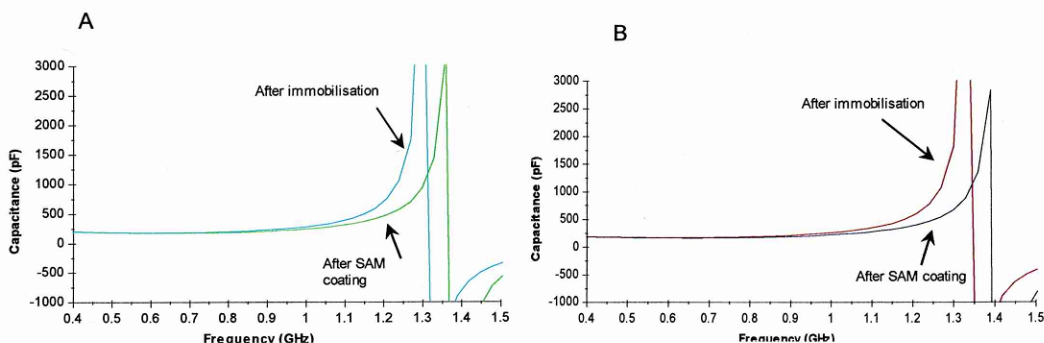


Figure 4.7. Capacitance measurements of two IDEs, number 1(A) and 2 (B) before and after anti-PSA antibody immobilisation. Measurement taken using a network analyzer and a probe station.

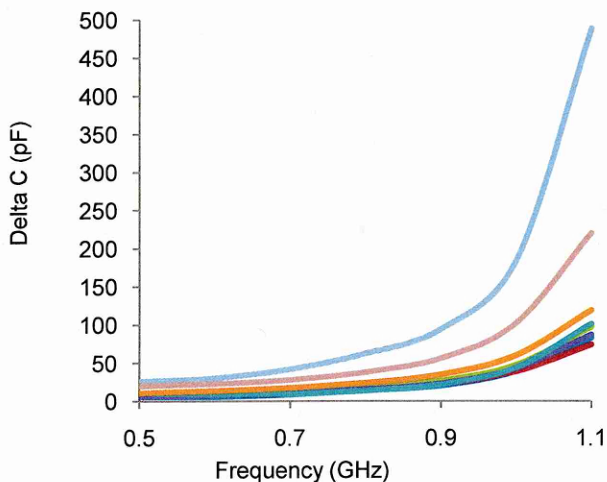


Figure 4.8. Capacitance measurements of 6 IDEs were measured before and after antibody immobilisation and the results were subtracted from each other to obtain the capacitance change due to surface modification.

After antibody immobilisation, PSA at varying concentrations were bound to the capacitor surfaces and again capacitance changes were measured. For each concentration of PSA three IDEs were used. As seen from Figure 4.9 and Figure 4.10, the capacitance measurements of the IDEs coated with epoxy silane and MUDA (without silane blocking) were not correlated with the PSA concentration.

Additionally the data points for each concentration of PSA showed varying results and not consistent within each other.

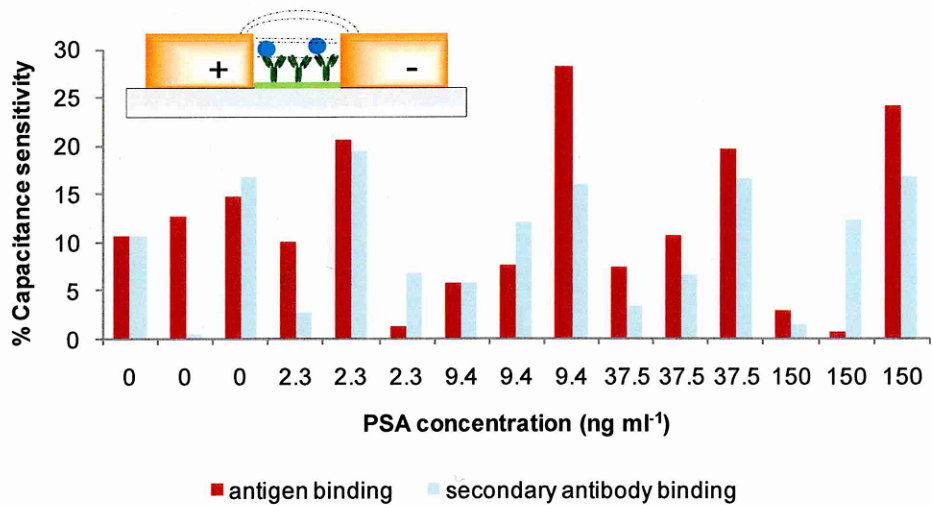


Figure 4.9. Silicon wafer A: Silicon oxide surface was modified with epoxy silane and Au electrodes were left blank. The plot shows both antigen (PSA) and secondary antibody (PSA detection antibody) binding responses of the capacitor measured at 2.8 GHz.

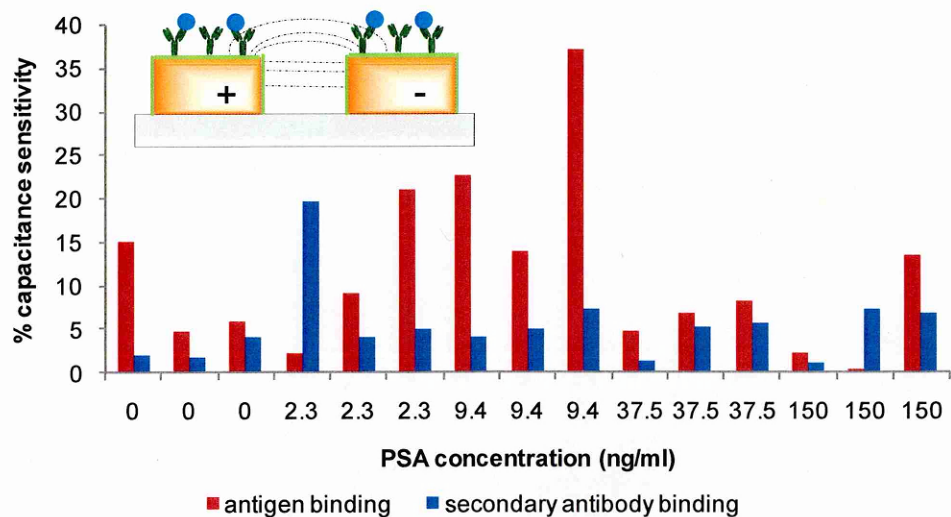


Figure 4.10. Silicon wafer B: Au electrode was modified with alkanethiol (MUDA) and silicon oxide layer between the electrodes were left blank. The plot shows both antigen (PSA) and secondary antibody (PSA detection antibody) binding responses of the capacitor measured at 2.8 GHz.

Out of three wafers with different surface chemistries, only wafer C (gold: MUDA, SiO₂: blocked with ethoxy-silane) gave reasonable results; the capacitance sensitivity change was correlated with the increase in PSA concentration (Figure 4.11). Although this result indicated that immobilisation of the antibody on to alkanethiol modified Au electrode and blocking the area between the electrodes with a blocking silane was the best method to get results from the IDE array, the coefficient variation (CV) of the data points were as high as 159%. To investigate if poor reproducibility was the result of the assay method or the weaknesses of the measurement using the probe station, further investigation was conducted as described in the next section.

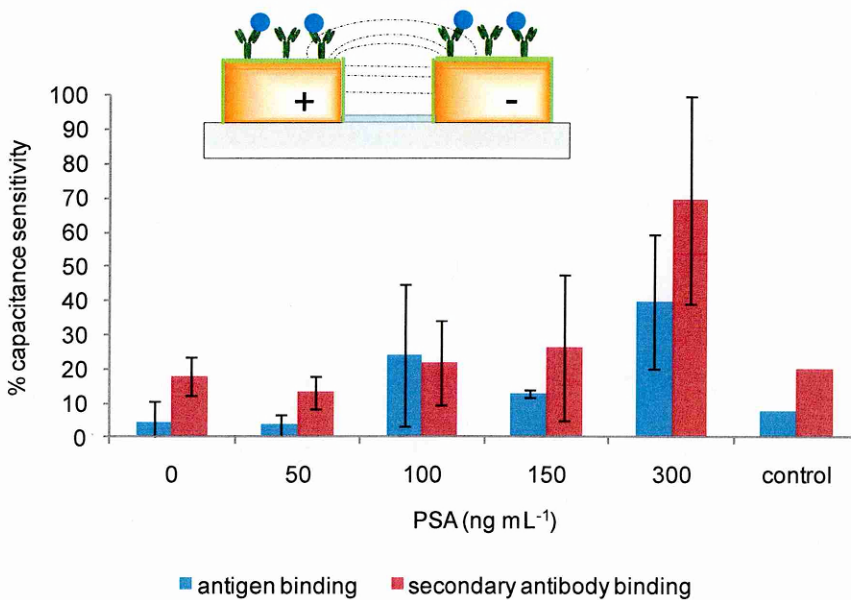


Figure 4.11. Silicon wafer C: Au electrode was modified with alkanethiol (MUDA) and silicon oxide layer between the electrodes were blocked with tetra-ethoxy silane. The plots show both antigen (PSA) and secondary antibody (anti-PSA) binding responses of the capacitor measured at 2.8 GHz. (n=3)

4.3.1.1 Investigation of the possible problems with the capacitance measurement using network analyzer and probe station

The reproducibility of the capacitance measurements using probe station was tested by measuring the capacitance of each capacitor by two users separately. In the previous section it was already shown that the standard deviation of capacitance measurements between the IDEs were quite high although same person used the probe station for measurements; in this occasion the objective was to find out if the capacitance measurement was also ir-reproducible if performed by different users. As seen from the Figure 4.12, the measurement difference between the users were up to 119 pF, indicating that capacitance measurement using the probe station was also highly dependent on user. Possible reasons to the variability of measurements might be:

- The pressure applied to the probe affects the reading from the network analyzer.
- The touch of probe damages different areas of SAM at each measurement which can alter the reading at subsequent measurements.

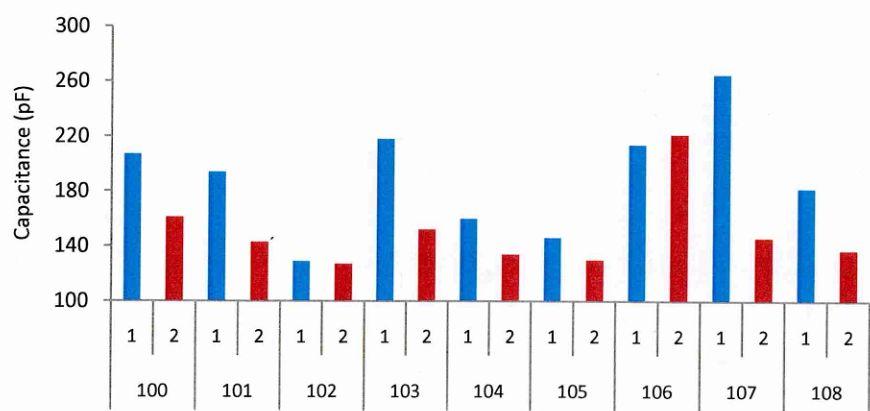


Figure 4.12. Capacitance of each interdigitated electrode of the array was measured by two users.

The poor reproducibility of capacitance measurements has been stated as a common problem by a review paper published on 2001 by Berggren and colleagues: “.....reproducibility constituting a major problem. A *significant amount of sensors did not give any signal at all* and for the others the *reproducibility was around 30 ± 60 %*. It is believed that the problem mainly is due to the *immobilization of the biorecognition elements*. If it is not compact and insulating, the responses will be low or not seen at all, but on the other hand the capacitance must be high in the insulating layer in order not to dominate the total capacitance.” (Berggren, Bjarnason and Johansson, 2001)

In a recent paper in 2009 Carrara and colleagues has described the problems with the capacitor sensors and this indicates that the problems with capacitive biosensors do still continue: “The ideal capacitance behaviour of a sensing probe has to be stable, sensitive and highly specific. Unfortunately, published data usually present a large time drift, resulting in a large standard deviation, largely scattered data points, poor reproducibility from electrode to electrode, and interface behaviour not so close to that of an ideal capacitor.” (Carrara et al., 2009)

Since the measurement using probe station was not resulted in reproducible and high quality reading, new IDE array capacitor and connector needed to be designed to overcome this problem. Additionally, measurement using probe station prevents the use of any buffer during the measurement, and with the new design this problem also needed to be solved. The quotation above from the review of Berggren et al. suggests that the quality control of surface chemistry is essential to obtain good quality and highly insulated sensor surface that has immense affect on the reproducibility of the

results. Therefore, new IDE array capacitor design also need to allow cyclic voltammetry measurements as a quality control measure for the coated surface of the capacitors.

4.3.2 Development of a new sensor chip and the connection unit

In this part of the study, interdigitated electrode arrays were designed, fabricated and used as a capacitive label-free biosensor for cancer biomarker detection. Interdigitated electrodes (IDE) used in the study consist of two arrays of electrodes with comb shape. Length of each electrode is $750\ \mu\text{m}$, width is $25\ \mu\text{m}$ and the distance between two electrodes is $25\ \mu\text{m}$. An array containing nine IDEs were fabricated with conventional microfabrication technologies on standard glass slides in a class 1000 clean room (Figure 4.13).

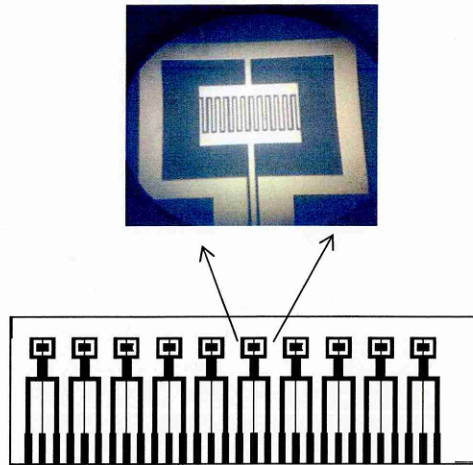


Figure 4.13. Interdigitated capacitor array design on a glass slide.

IDE fabricated glass slide was connected to a 41R LCR meter (CHY Firemate C., Taiwan) with help of a PCB. The PCB contained nine switches and enabled measurement of one capacitor at a time. The capacitance of each part of the custom

prototype was measured to assess the effect of the PCB and connectors to total capacitance measured. Also the capacitance of dry and buffer (PBS) dipped electrodes were measured for comparison (Figure 4.14). As seen from the Table 4.1, there was big difference (20,000) in capacitance between wet (nF range) and dry IDEs (pF range). The capacitance measurement while IDEs dipped in PBS resulted in around 40 nF capacitance, and as it was around 3000 times higher than the total capacitance of the PCB and connectors, the only influence to the capacitance would be the changes in the surface properties of IDEs. Therefore, capacitance measurements performed while IDEs dipped in PBS for the further experiments.

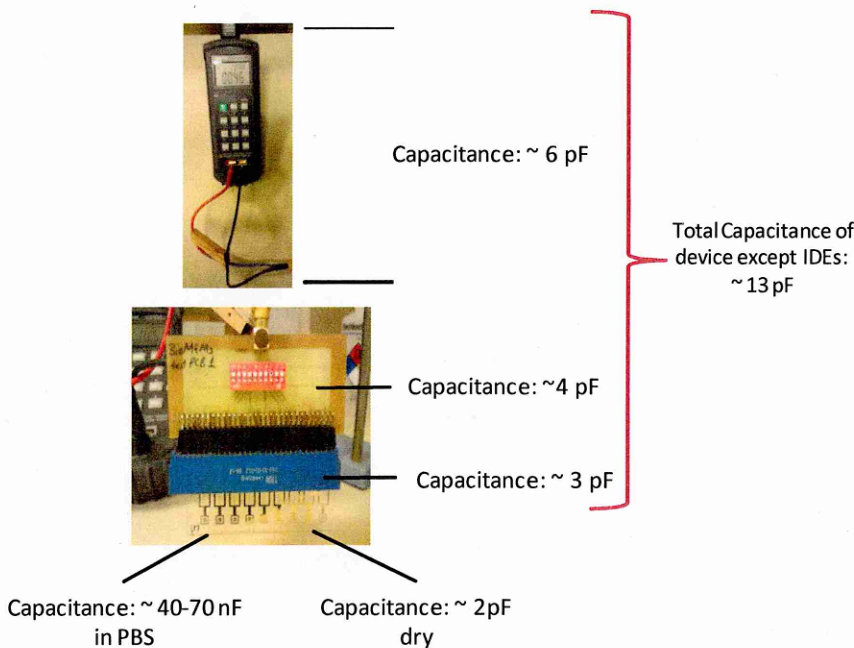
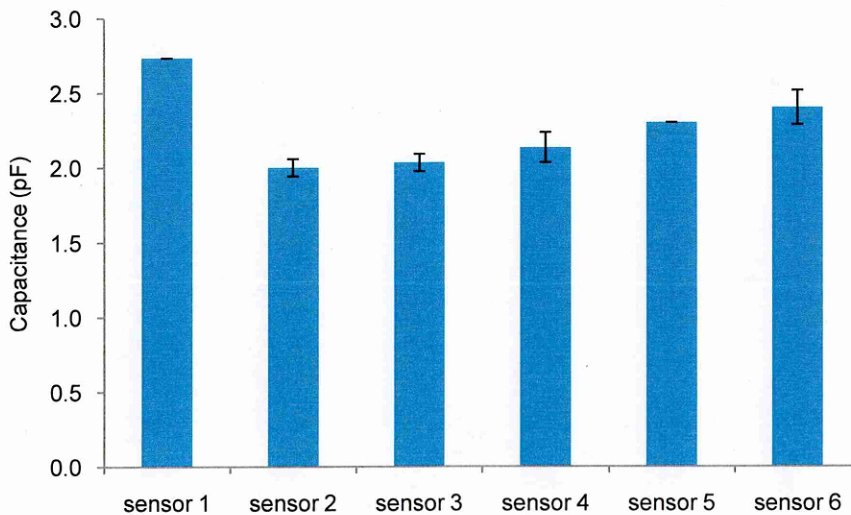


Figure 4.14. Capacitance measurements of different parts of the biosensor platform developed. Capacitances of IDEs were measured in dry or wet (IDEs dipped in PBS) conditions.

Table 4.1. The summary of the capacitance measurements obtained from the parts of custom prototype.

	Wet measurement	Dry measurement	Difference
Total capacitance of all connectors	~ 13 pF	~ 13 pF	-
Capacitance of IDE	Min. 40 nF	~ 2pF	20,000 times
IDE capacitance to connector capacitance ratio	3077	0.15	20,000 times

The capacitance measurements using network analyzer and probe station suffered from irreproducible measurements. For comparison, the reproducibility of the capacitance readings were tested using newly developed custom prototype. The variation between the consequent measurements of the same capacitor (Figure 4.15) and the measurement differences between different capacitors (Figure 4.16) were minimal.

**Figure 4.15.** The capacitance of each sensor on a glass slide was measured at 1 kHz using an LCR meter.

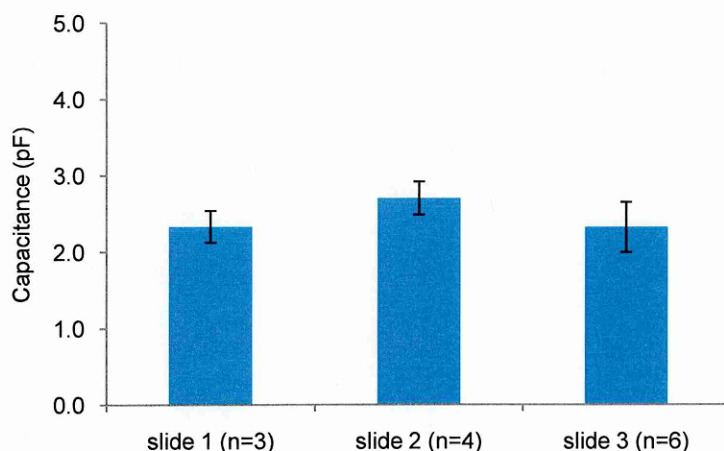


Figure 4.16. Capacitances of all the sensors in three glass slides were measured at 1 kHz using an LCR meter.

4.3.2.1 IDE surface chemistry

The newly designed Au electrode surfaces were first modified with an alkanethiol monolayer and glass slide was modified with a blocking silane to prevent non-specific binding of antibody and antigen to the surface. Cyclic voltammetry was used to characterize the SAM coated IDEs. Cyclic voltammetry measurements were performed with a BAS Electrochemical Analyzer. In this study 1 mM potassium ferricyanide prepared in 1M KCl was used as an electroactive marker for cyclic voltammetry measurements. The gold electrode surface of the IDE forms the working electrode. After the IDEs dipped in to potassium ferricyanide solution, for reference Ag/AgCl wire and for counter electrode platinum wire were used. Scan for potassium ferricyanide was set to 3 cycles from 0 to 0.5 V.

When an electrode is modified with SAM, insulating properties of the monolayer causes the peak current to decrease. If the alkanethiol molecule used for self assembly contains 10 or more alkyl changes (as in the case of MUDA that is used in

this study), a very compact insulating layer is formed on the electrode surface and hence the cyclic voltammogram adopts a sigmoidal line shape as microelectrodes. The reason of this microelectrode resemblance of the cyclic voltammogram is the imperfections of the monolayer (pinholes) where electron transfer reaction occurs (Finklea et al., 1993; Janek et al., 1998). More information on the quality control of surface chemistry using cyclic voltammetry can be found from the Sections 2.2.2.3 and 2.2.3.2. As seen from Figure 4.17, the surface coverage of MUDA coated sensors/IDEs were in the range of $\sim 99\%$, which provided the perfect insulating properties and was necessary for further capacitive biosensor measurements.

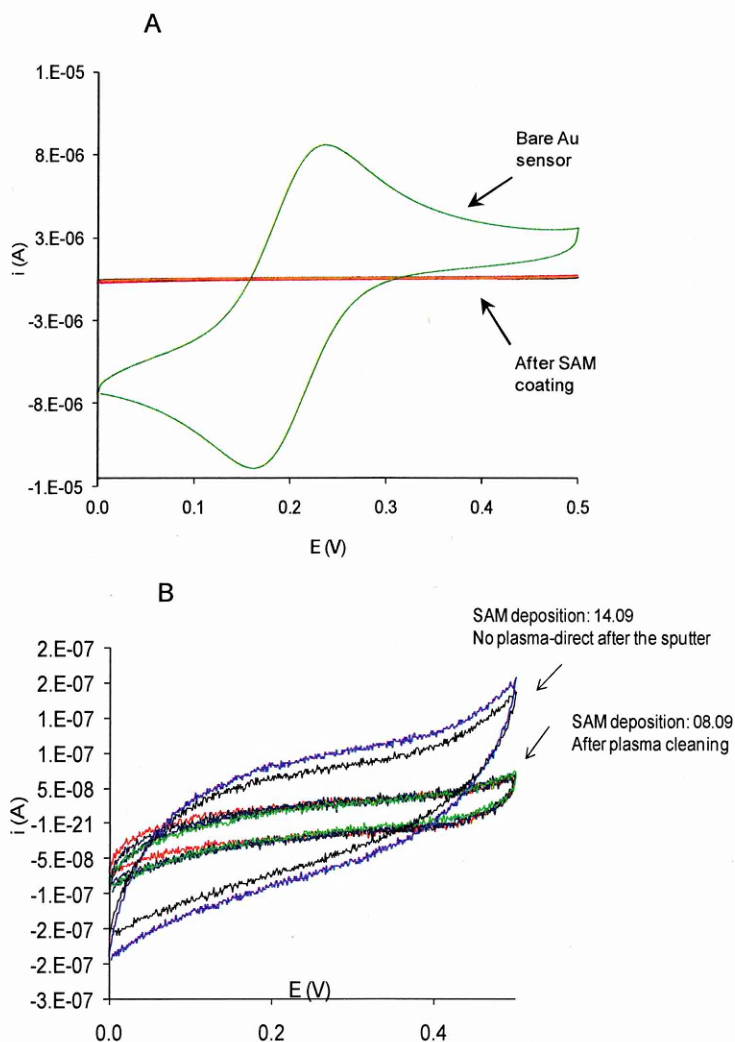


Figure 4.17. A) Cyclic voltammograms for bare Au and SAM coated sensors were measured using a potentiostat with 1 mM potassium ferricyanide in 1 M KCl. B) The cyclic voltammogram of SAM coated sensors were zoomed out. The surface coverage was calculated as 98 % for the IDEs coated with SAM after metal sputtering process. The surface coverage was calculated as 99 % for the IDEs that were plasma cleaned prior to SAM deposition.

4.3.2.2 Continuous assay for the detection of PSA using capacitance

The measuring principle of capacitance biosensor developed was based on the measurement of changes in dielectric properties and charge distribution due to

antibody-antigen complex formed on the surface of an electrode. The capacitance (C) can be described by equation 4.2; where ϵ is the dielectric constant of the medium between the plates, ϵ^0 is the dielectric constant of free space (8.85419 pF), A the area of the plates and d the distance between the plates.

$$C = \epsilon \times \epsilon^0 \times A/d \quad \text{Equation 4.2}$$

As can be seen from the Equation 4.3, the capacitance of the biosensor consist of several capacitors in series (Berggren et al., 2001); the capacitance of the SAM layer that insulates the electrode surface, the capacitance of the recognition layer (antibody immobilised electrode surface) and the capacitance of the diffuse layer formed by the buffer contacting the electrode.

$$\frac{1}{C_{total}} = \frac{1}{C_{SAM \text{ layer}}} + \frac{1}{C_{recognition \text{ layer}}} + \frac{1}{C_{diffuse \text{ layer}}} \quad \text{Equation 4.3}$$

From Equation 4.3 it can be seen that the lowest capacitance dominates the total capacitance. To prevent the capacitance of the SAM layer affecting the total capacitance, the SAM layer formation was optimised to obtain maximum surface coverage and insulating properties. To control the surface coverage properties cyclic voltammetry experiment was performed using potassium ferricyanide as the oxidation/reduction reagent. By comparing uncoated and SAM coated electrodes it was calculated that surface coverage was around 99%. As the surface insulation is close to perfect, we could assume that total capacitance would be dominated by the capacitances of the recognition layer and diffuse layer. Antigen binding to the sensor surface changes the dielectric properties of the sensor surface and the increased

thickness of the layer would decrease the capacitance of the recognition layer (Carrara et al., 2009; Jiang et al., 2003). Additionally, bound antigen will displace the ions of the double layer formed in the diffuse layer and causes a drop of the $C_{\text{diffuse layer}}$ (Novak, 2007). In short the total capacitance of the biosensor decreases due to antigen binding to the capacitor biosensor and this change is measured for antigen detection.

Capacitance measurements were taken using a 41R LCR meter from CHY Firemate C. (Taiwan). The LCR meter was connected to the computer to record the capacitance results every 10 seconds to obtain continues assay trace (Figure 4.18). The IDE was dipped to buffer (10mM PBS, pH 7.4) then capacitance measurements were taken to provide a baseline. Later the immobilisation and antigen binding was performed by changing the buffer solution with the required solutions and the capacitance was continued to be measured every 10 seconds (Figure 4.19). After obtaining continues assay curve the capacitance drop due to immobilisation and antigen binding was calculated by simply subtracting the capacitance before and after the immobilisation/binding. PSA antibody immobilisation resulted in 17.9 ± 1.8 nF ($n = 3$) capacitance change. Antigen binding assays were then developed to capture the PSA molecule in buffer solutions achieving a detection limit of 15.6 ng mL^{-1} with a linear dynamic detection range of $15.6 - 250 \text{ ng mL}^{-1}$ PSA (Figure 4.20).

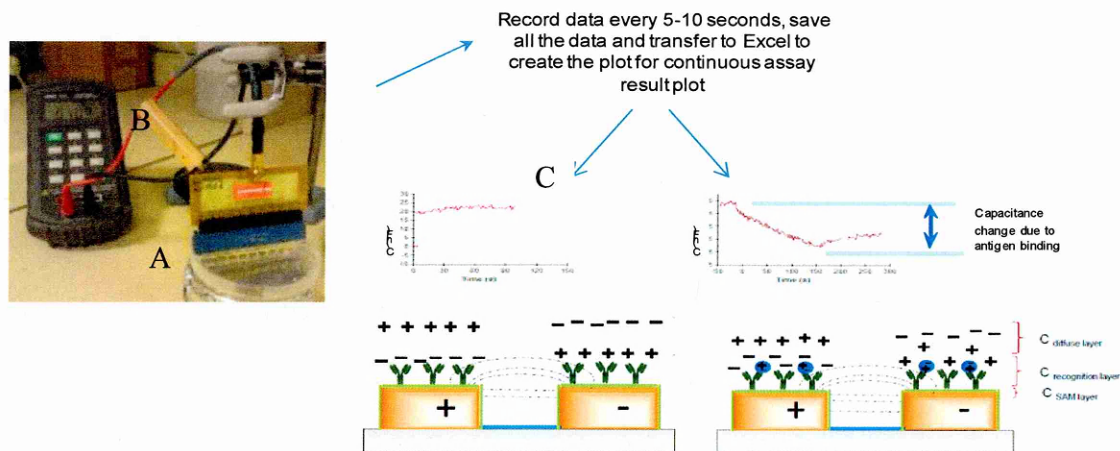


Figure 4.18. The schematics of a capacitive biosensor assay. The interdigitated electrode used was dipped to a buffer solution (PBS) to get a baseline reading, and then the buffer was changed to assay solutions such as antibody for immobilisation or antigen for binding (A). The LCR device was connected to a computer (B) and the readings were taken every 10 seconds throughout the assay process to create a continuous assay plots (C).

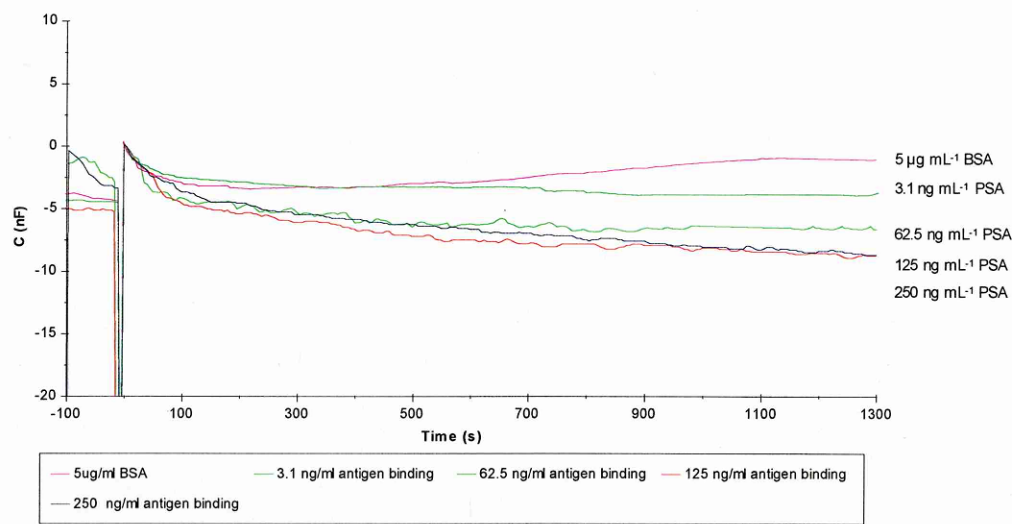


Figure 4.19. Continuous assay trace for binding of PSA to the PSA capture antibody immobilised sensor surface.

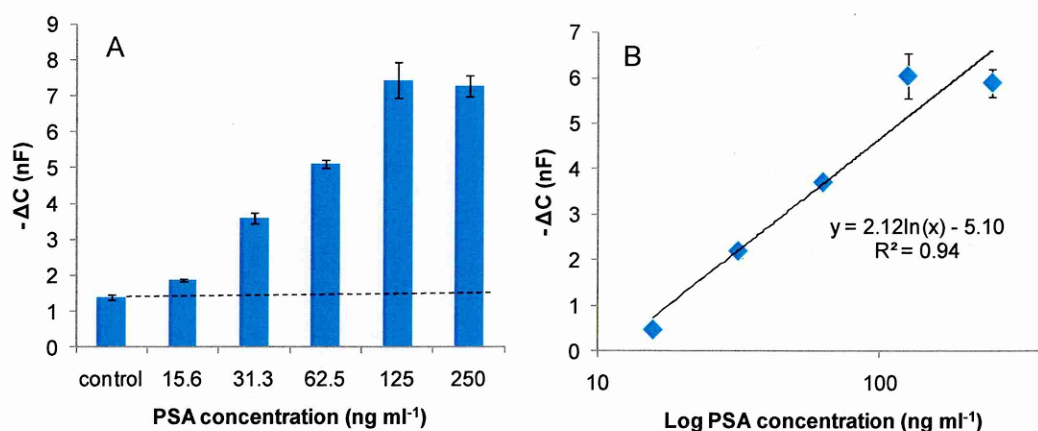


Figure 4.20. Capacitance changes due to PSA binding to the anti-PSA antibody immobilised IDEs were plotted with (B) and without (A) control subtraction.

4.3.3 Sensor chip fabrication process

The quality of the IDEs was checked while fabrication process, to ensure a high quality sensor chips fabrication. For that purpose the microscope images of an IDE was taken after photoresist application and after sputtering with Au (Figure 4.21). Both images showed some dark spots and scratches on the surfaces. These indicated that the piranha cleaning (sulphuric acid: hydrogen peroxide, 3:1) process applied to the glass slides prior to lithography process was not efficient enough. There could be two reasons for that; the piranha cleaning process was not performed in a heated sonication bath that might have enhanced the cleaning process, and secondly the reagents might be contaminated or shelf lives expired. A short test was also performed to find out the effect of piranha cleaning to the glass slides. Therefore a glass slide was sputtered with Au without any prior cleaning and another glass slide was piranha cleaned prior to metal sputtering. As seen in Figure 4.22, while the uncleaned glass slide surface was full of large spots, the piranha cleaned surface had very few spots. This test clearly showed that the glass slides needed to be cleaned

effectively prior to lithography process. As the test showed, to fabricate high quality IDEs, piranha cleaning was required, however this cleaning process need to be improved. Therefore, it was decided to purchase smaller bottles of hydrogen peroxide that would be used fresh. Additionally cleaning in a sonication bath would be considered.

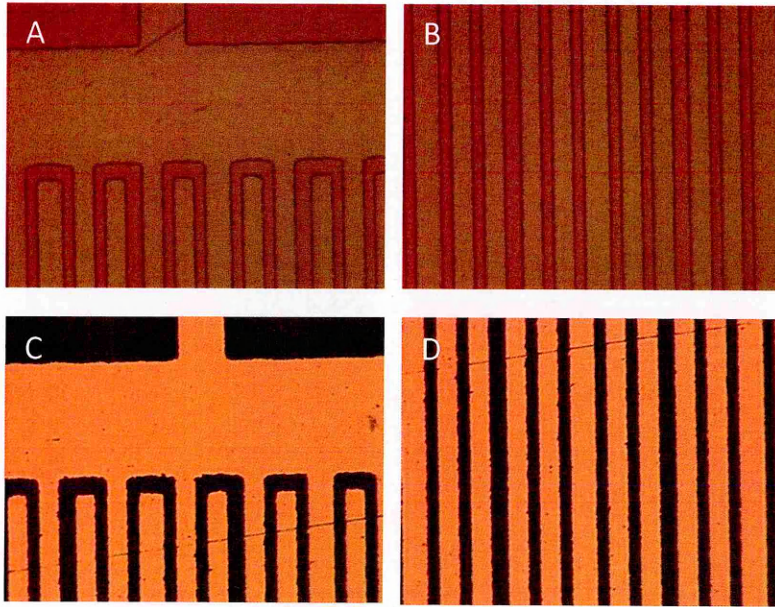


Figure 4.21. The images of interdigitated electrode after photoresist (A and B) and Au (C and D) coating. Both surfaces had spots and scratch marks.

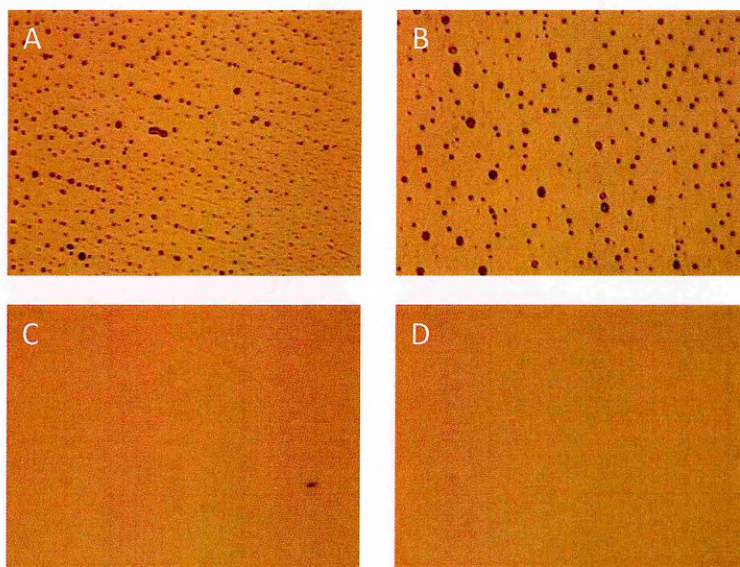


Figure 4.22. One of the glass slides were coated with Au without any prior cleaning process (A and B) and the other glass slide was sputtered Au after cleaning with piranha solution (sulphuric acid: hydrogen peroxide, 3:1) (C and D).

4.4 CONCLUSIONS

Initially interdigitated capacitor biosensors fabricated on silicon wafers were used as sensors and capacitance detection was performed using a network analyzer and a probe station. Although several experiments were performed, due to variable and irreproducible results, it was decided to change the design of the capacitor sensors and the detection instrument used to an LCR meter. Later a new interdigitated electrode array and PCB were designed and fabricated, and the capacitance reading was performed using a simple LCR meter. This new prototype enabled both consisted capacitance reading and also enabled the detection to be performed in buffer. Antigen binding assays were developed to capture the PSA molecule in buffer solutions achieving a detection limit of 15.6 ng mL^{-1} with a linear dynamic detection range of $15.6 - 250 \text{ ng mL}^{-1}$ PSA.

CHAPTER V:

FINAL DISCUSSION AND CONCLUSION

5 FINAL DISCUSSION AND CONCLUSION

This thesis covers the development of surface chemistries for sensors platforms and an affinity sensor for prostate cancer diagnosis using PSA as the protein biomarker in blood samples. Self assembled monolayers, PAMAM dendrimers, CMD and a new novel polymer surface developed during the study were considered to immobilise biomolecules to sensor surfaces. A patent application was registered to protect the intellectual property of the developed novel polymer surface. Additionally QCM and SPR sensors were utilised for prostate cancer diagnosis and Au nanoparticles were used to enhance the sensitivity of the PSA detection tests. A new buffer was designed to perform the tests in human serum with minimum matrix effects that enabled test of patient samples from Bedford Hospital. This thesis has demonstrated the applicability of different surface chemistries for sensing and in particular introduced a novel polymer surface for biomolecule immobilisation. In addition this study showed the suitability of the developed immunoassay for fully automated QCM and SPR sensors for patient sample testing as an alternative to conventional ELISA type tests for prostate cancer.

5.1 SURFACE CHEMISTRY DEVELOPMENT FOR SENSORS FABRICATION

5.1.1 Self assembled monolayer deposition optimisation

The type of surface chemistry used to immobilise biomolecules to sensor chip is crucial to obtain good detection signal from the device. In the current study initially SAM surface preparation protocol were optimised and the quality of the surface was

tested using cyclic voltammetry, contact angle and AFM measurements. The cleaning of the gold sensor surface is critical before the application of alkanethiol adsorption. The minute amount of impurities on the gold surface prevents the attachment of sulphur atoms to the gold atoms and prevents the formation of well packed monolayer. The pre-treatment of the surface may consists of electrochemical cleaning, chemical cleaning, plasma cleaning or UV/ozone irradiation. For the cleaning of gold sensor surfaces two approaches were considered as ethanol wash and plasma cleaning. While a very high contact angle was observed after ethanol wash indicating the surface was not clean enough, water contact angle of ca. 14 degrees was obtained after nitrogen plasma indicated that the gold sensor surface was clean enough to apply SAM deposition.

In a study Raiber *et al.* used hydrogen and oxygen plasma to obtain a clean gold surface for SAM application (Raiber *et al.*, 2005). While they obtained 0 degrees contact angle after 20s of oxygen plasma, 70 degrees was obtained after hydrogen plasma cleaning process. The authors' comment that, the unexpected high contact angle of hydrogen plasma could be due to the attraction of hydrocarbons to the clean surface and instantly increasing the contact angle and this phenomenon has been seen at other studies as well. In the case of oxygen plasma, an oxide layer on the gold forms and this layer quickly oxidizes hydrocarbons and keeps the surface hydrophilic. In another study, Min *et al.* compared three different cleaning procedures; super-piranha cleaning [(1:10:6) 61% HNO_3 : 30% H_2O_2 : 95% H_2SO_4 (v/v/v)], piranha cleaning [(1:4) 30% H_2O_2 : 98% H_2SO_4 (v/v)] and 5 minutes sonication of gold substrates in ethanol (Min *et al.*, 2008). The contact angle measurements showed that the lowest contact angle measurement was obtained as 60

degrees when super-piranha cleaning method was utilised. High contact angle after super-piranha cleaning indicated that cleaning performance of piranha or super-piranha cleaning process was not better than plasma cleaning that was used in this study. Care needs to be taken while using piranha solution as it is very reactive and dangerous. With respect to piranha cleaning or any other chemical cleaning methods, plasma cleaning is simple, easy and use of dangerous chemicals are not required. Tsai *et al.* compared three different cleaning procedures; O₂ plasma cleaning, O₂ plasma cleaning followed with ethanol rinse and O₂ plasma cleaning followed with 1 hour sonication in ethanol; and the contact angle of water obtained using these methods were $9.3^\circ \pm 2.8^\circ$, $18.8^\circ \pm 2.5^\circ$ and $77.9^\circ \pm 10.7^\circ$ respectively (Tsai and Lin, 2001). Low contact angles obtained using the first two methods showed the effectiveness of the oxygen plasma cleaning method. However oxygen plasma creates a gold oxide layer on the substrate and this layer needs to be reduced completely with ethanol prior to SAM deposition. In conclusion, the obtained water contact angle of ca. 14 degrees after nitrogen plasma cleaning indicated that the gold sensor surface was clean enough to apply SAM deposition and the result was comparable with the studies in the literature. Later, cyclic voltammetry method was used to assess the quality of the formed monolayer after nitrogen plasma cleaning.

Application of SAM on a gold electrode surface insulates the electrode surface. By comparing the cyclic voltammograms of a bare gold electrode and alkanethiol coated gold electrode, it is possible to investigate the coverage of the monolayer on the gold electrode. For example, Campuzano *et al.* used a gold disk electrode that has 0.0585 cm² geometrical area and calculated surface coverage as 0.997 for an MUDA coated electrode (Campuzano et al., 2006). In our study the electrode area of QCMA-1

sensor chips were ca. 0.42 cm^2 and the obtained surface coverage was 0.971, indicating a very compact SAM with respect to the electrode area used for cyclic voltammetry and this indicates that SAM was very compact and the optimised gold cleaning procedure and monolayer deposition technique was efficient.

5.1.2 Use of PAMAM dendrimers to enhance surface capacity

To be able to increase the capacity of the sensing layer as an alternative to SAM surface, dendrimers were considered. PAMAM dendrimers are large, branching molecules formed by repeated addition of functional groups to an ethylenediamine core. Dendrimers possess a large number of end groups per molecule and thereby increase surface functionality (Rahman et al., 2000) and in the study sensing on dendrimer surface using QCM and SPR sensors were tested to see if the physical capacity increase of the sensor surface would be transferred to the detection signal. It was found that dendrimer surface did not increase the capacity of the sensor surface if used for QCM biosensor with the current format (NeutrAvidin capture assay on biotinylated dendrimer surfaces); however protein binding capacity of the sensor increased 1.5 times if it was used for an SPR biosensor.

In an earlier study in 2004, Mark et al. suggested that dendrimer modified surfaces caused a 2.5 fold antibody immobilisation signal enhancement with respect to a flat SAM surface (Mark et al., 2004). Two recent studies by Singh et al. and Shayir et al. has shown 1.6 times and 2 times higher immobilisation levels using dendrimer coated surfaces, respectively (Singh et al., 2009; Syahir et al., 2009). In our study we observed 1.5 times higher immobilisation levels using dendrimers that was close to the results obtained in recent studies. In conclusion, dendrimer monolayers can be used to increase the sensor surface capacity ca. 1.5 times for SPR sensor chips

however dendrimer surface has no effect to increase for QCM sensor surface capacity.

5.1.3 Development of a novel polymer surface (UVlink) for biomolecule immobilisation on sensors

In the present study a polymeric structure was grafted on the substrate surface to provide a new sensor surface prior to biomolecule immobilisation. To graft polymer layers on solid matrices, free radical chain polymerisation is a widely used method. The prepared sensor surface was tested on both SPR and QCM sensors. Several polymer surfaces were fabricated on QCMA-1 sensor chips and used for biomolecule immobilisation and further binding assays. The results indicate that polymer surface may not be suitable for QCMA-1 sensor chips. The QCM device is very sensitive to the rigidity of the polymer surface grafted on the sensor surface. It is very difficult to obtain a rigid surface (that is required to obtain a good QCM sensing) while increasing the pore size of the polymer for improving biomolecule immobilisation capacity.

Polymerisation process has been optimised and polymer coated sensors have been tested using an SPR biosensor. Visible and uniform polymer surfaces were fabricated on SPR sensor chips having different surface properties (flat or uniformly patterned surface) and biological tests were performed using Biacore 3000 instruments. The polymer surface has similar antibody immobilising but higher antigen binding capacity with respect to MUDA coated SPR chips; higher immobilisation capacity but slightly lower antigen binding capacity with respect to a Biacore C1 sensor chip. The main advantage of polymer coated sensors with respect to MUDA and C1 sensor chips is the longer shelf life. While the shelf life of MUDA or C1 sensor chips are

around 4 to 6 months maximum, theoretically the shelf life of our polymer surface is minimum a year. Additionally different monomers (such as biotin or NTA functionalised) can be utilised to change the functional group of the polymer to achieve different type of surfaces. A patent application was filed for the work in July 2009 (Y. Uludag and I. E. Tothill, High capacity polymer surface for immobilisation of biomolecules', Date Lodged: 10 July 2009, GB0911634.4) and this was re-filed in July 2010.

5.1.4 Development of a carboxymethyl dextran surface

CM5 sensor chip developed by Biacore-GE Healthcare has a very high biomolecule immobilisation capacity with respect to monolayer surfaces and till recently was protected by a patent (US Patent No: 5,242,828). As this patent was recently expired, the use of this surface for SPR-2 instrument sensor chips (Sierra Sensors GmbH) was investigated. Two different fabrication methods so-called amide-dextran and epoxy-dextran methods were applied and both methods resulted in a CMD surface on sensor chips. A functional immunoassay was applied to the fabricated Biacore 3000 and SPR2 sensor chips to investigate the performance of the coatings. It was observed that the functional assay responses obtained from the epoxy-dextran coated Biacore 3000 sensor chips and purchased CM5 sensor chips were close to each other but the surface fabrication procedure need to be improved to achieve the same antigen binding results. The same protocol was applied to SPR2 sensor chips and in general the functional assay responses were lower with respect to the responses obtained from Biacore 3000 sensor chips. This could be related to the size of the SPR2 sensor chips which might have influenced the coating process. Additionally the flow cell size of SPR2 instrument is larger than the Biacore 3000 instrument and in-efficient washing of the sensor chip after dextran coating cause flow disturbances which

might have influenced the results. It is expected to obtain better results from the dextran coated SPR2 sensor chips after the washing steps of the dextran coating procedure was improved. As the molecular weight of the CMD used for amide-dextran (MW 10,000 – 20,000) sensor coatings were lower in comparison to the epoxy-dextran (MW 500,000) method, the immobilisation of antibodies to this sensor surfaces produced lower responses with respect to epoxy-dextran coated sensor chips. However the antigen binding responses obtained using Biacore 3000 instrument was very close to the epoxy-dextran coated sensor surface. Again SPR2 instrument produced lower responses from the immunoassay applied on amide-dextran coated sensor chips. However the reason of this lower response could have similar reasons as described above for the epoxy-dextran coated SPR2 sensor chips. In conclusion, both amide-dextran and epoxy-dextran methods are suitable for CMD coated sensor chip fabrication.

5.2 DEVELOPMENT OF AN AFFINITY SENSOR FOR PSA DETECTION

Early diagnosis of cancer biomarkers is essential for successful treatment of the disease. The current biomarker tests are ELISA type and usually performed at centralised labs using automated devices. In this work, an investigation was described for the use of biosensors to perform cancer biomarker detection test as an alternative to conventional ELISA type tests. In the study, as cancer biomarker PSA was chosen and PSA test was developed and optimised using QCM and SPR devices to perform tests to the patient samples. Initially the PSA binding to the PSA capture antibody immobilised surface was tested in a concentration range between 4.7 to 150 ng mL⁻¹ and PSA binding response curves were then fitted to 1:1 Langmuir binding model to determine the binding association and dissociation rates, from which K_D value was calculated as 9.46×10^{-10} M using SPR instrument and 5.56×10^{-10} M

using the QCM sensor. Comparing these results with those achieved by other authors indicate good sensitivity of the assay with the used antibody. Karlsson *et al.* calculated the affinity of anti-PSA antibody towards PSA as 3.3×10^{-9} M (antibody from Fitzgerald Industries Int., clone M212091) (Karlsson *et al.*, 2006). Katsamba *et al.* calculated the affinity as 1.1×10^{-9} M (antibody from Fitzgerald Industries Int., clone M612166) (Katsamba *et al.*, 2006).

A new buffer was formulated to eliminate 98% of the non-specific human serum protein binding to the sensor surface. In a recent study, Situ *et al.* achieved 94% reduction in non-specific binding by using CM5 (carboxymethyl dextran) surface and a formulated buffer together and Trevino *et al.* achieved 88% reduction in non-specific binding of serum by using another formulated buffer (Situ *et al.*, 2008; Trevino *et al.*, 2009). With use of the developed matrix buffer, a sandwich assay using QCM and SPR affinity sensor chips resulted in detection of tPSA concentrations as low as 0.29 ng mL^{-1} in 75% human serum. The developed assay has some advantages including; short assay time, repeated usability of the same sensor chip, ability to detect PSA in high serum concentrations and utilisation of label-free detection method. This enables the optimised assay format to be a promising tool for clinical diagnosis/prognosis.

The number of publications related to the detection of PSA using QCM biosensor is very limited and this indicates that application of QCM biosensors to PSA detection is a newly developing area. An example to a previous study for PSA assay using QCM biosensor has been the study performed by Zhang *et al.* which involve the use of an insert-plug model of piezoelectric immunosensor. Although the PSA detection

limit obtained from this study was low enough (1.5 ng mL^{-1}), the reaction time was long and assay needs to be performed at elevated temperatures and additionally the dynamic range of the assay was limited (Zhang et al., 2007a). In another study, Ding *et al.* used yeast cell modified QCM sensors to immobilise PSA antibodies (Ding et al., 2008). With this method Ding *et al.* achieved detection of PSA in the range of 5 to 604 ng mL^{-1} in 100% serum. Both applications mentioned above were performed using non-automated equipment that due to their manual operation may not only cause variations between the assays but also are not suitable for clinicians use. The number of literature available for PSA tests performed using SPR instruments are more in numbers than the tests using QCM instrument. For example, Cao *et al.* performed 10 min PSA-ACT assay in 10 % human serum using an automated SPR biosensor and obtained a detection limit of 18.1 ng mL^{-1} that corresponds to LOD of 181 ng mL^{-1} for the whole serum (Cao et al., 2006). As a further assay Huang *et al.* used streptavidin modified gold nanoparticles to enhance the signal and obtained 1 ng mL^{-1} detection limit for PSA assay in buffer (Huang et al., 2005). Another study performed by Besseling *et al.* consists of two step signal enhancement process including sandwich assay with rabbit anti-PSA antibodies and further binding of goat anti-rabbit IgG modified Au nanoparticles (10nm) (Besselink et al., 2004). 30 minutes static assay using IBIS SPR biosensor (IBIS Technologies, Hengelo, Netherlands) resulted in LOD of 0.15 ng mL^{-1} for PSA assay in buffer. The experimental conditions vary between the assays significantly such as, assays in static or in flow, temperature, assay time and the antibody used against PSA. All these contribute to the assay sensitivity and hence make it difficult to compare the results effectively. If we compare the current study with published data briefly, as stated above after less than 10 minutes total reaction time, we obtained LOD of 0.29

ng mL⁻¹ in 75% human serum using both QCM and SPR instruments; indicating that our optimised PSA assay outperforms the assays performed by other researchers. Additionally due to it is being rapid and wide detection range the QCM and SPR immunoassay developed outperforms the commercial ELISA kits available for biomarker detection

In the study incorporation of fPSA, cPSA and PSMA immunoassays to the prostate cancer detection test was considered to enhance the predictivity of the test. For this purpose PSMA, fPSA and cPSA antibodies were purchased from two companies and their performances were investigated. fPSA and PSMA antibodies did not work for the assay at all and the affinity of both cPSA antibodies were very low and not suitable for the assay. This result clearly shows that the performance of the biosensor tests depend on the quality of the antibodies used for the immunoassay. The antibody producing companies tend to perform quality control protocols using gels or ELISA tests; however these tests do not guarantee that the same antibody will perform well for biosensor immunoassays. This causes problem while selecting the right antibodies for the biosensor assay development as seen from the current study.

Patient samples collected from Bedford Hospital and control samples collected from Cranfield University staff were tested and it was found that the developed QCMA-1 test clearly discriminates the PSA level of patients from the controls.

5.3 CAPACITIVE BIOSENSOR DEVELOPMENT FOR PSA DETECTION

A new interdigitated capacitive electrode array and PCB biosensor platform based on label-free interdigitated capacitive sensing principle developed by Sabanci

University (Istanbul, Turkey) was also employed in the study. IDE arrays were fabricated with conventional microelectronics-micromachining technologies on silicon wafers in a class 1000 clean room. Later IDE arrays were utilised for the detection of PSA using an LCR meter. This new prototype enabled both capacitance reading and also enabled the detection to be performed in buffer solution. Antigen binding assays were developed to capture the PSA molecule in buffer solutions achieving a detection limit of 15.6 ng mL^{-1} with a linear dynamic detection range of $15.6 - 250 \text{ ng mL}^{-1}$ PSA.

5.4 FINAL CONCLUSION

The overall results achieved from the study include the development of fabrication protocols for different sensor surface chemistries and this included a novel sensor surface fabrication. Additionally PSA immunoassay was performed successfully using biosensors to detect PSA not only in buffer but also in patient serum samples at clinical levels.

Overall conclusion of the performed study is listed below:

Sensor surface chemistry development:

- SAM deposition protocol was optimised and quality control methods were developed for surface. In the study the electrode area of QCMA-1 sensor chips were ca. 0.42 cm^2 and the obtained surface coverage was 0.971.
- While the developed dendrimer surface may not increase the capacity of QCM sensor, it may enhance the capacity of SPR biosensors ca. 1.5 times.
- The developed novel polymer surface was synthesised and this may be used to immobilise biomolecules and has longer shelf life than conventional SAM layers and hydrogel surfaces. A patent application was filed to protect the intellectual property of the developed surface.
- CMD surface fabrication protocol was developed. This needs further optimisation in future work.

Development of an Affinity Sensor for PSA detection:

- An immunoassay was developed for PSA using a prototype QCM device and the results were compared using a commercial SPR biosensor.

- A new buffer called matrix buffer was developed that minimised 98% of serum protein non-specific binding to the sensor surface.
- The sensitivity of the developed assay was enhanced by employing Au nanoparticles. The summary of the assay results are shown in Table 5.1, Table 5.2 and Table 5.3.
- Patient samples collected from Bedford Hospital and control samples collected from Cranfield Health staff was tested and it was found that the developed QCMA-1 test clearly discriminates the PSA level of patients from the controls.

Table 5.1. Results of kinetic calculations for PSA direct assay and detection limits using SPR and QCM devices.

	SPR (Biacore 3000)	QCM (QCMA-1)
K_D	$9.46 \times 10^{-10} \text{ M}$	$5.56 \times 10^{-10} \text{ M}$
k_a	$2.08 \times 10^{+06} \text{ M}^{-1} \text{ s}^{-1}$	$1.23 \times 10^{+06} \text{ M}^{-1} \text{ s}^{-1}$
k_d	$1.97 \times 10^{-03} \text{ s}^{-1}$	$6.83 \times 10^{-04} \text{ s}^{-1}$
R_{max}	110 RU	108 Hz

Table 5.2. The summary of the PSA detection assay results obtained using QCM device (QCMA-1).

	direct assay in PBS/T	sandwich assay in PBS/T	sandwich assay in 75% serum	sandwich assay in 75% serum using 40 nm Au nanoparticles
LOD	18.8 ng mL ⁻¹	4.7 ng mL ⁻¹	9.4 ng mL ⁻¹	0.29 ng mL ⁻¹
Linear range	18.8 – 150 ng mL ⁻¹	4.7 – 150 ng mL ⁻¹	9.4 – 150 ng mL ⁻¹	0.29 – 150 ng mL ⁻¹
Equation	y = 0.87x + 0.27 (R ² = 1.00)*	y = 0.91x + 0.35 (R ² = 0.99)*	y = 0.89x + 0.33 (R ² = 0.98)*	y = 71.96Ln(x)+73.77 (R ² = 0.99)

*y = log (response); x = log (concentration)

Table 5.3. The summary of the PSA detection assay results obtained using SPR sensor (Biacore 3000).

		Direct assay	Sandwich assay with PSA antibody	Sandwich assay with PSA antibody modified 20 nm Au nanoparticles	Sandwich assay with PSA antibody modified 40 nm Au nanoparticles
PSA in buffer	LOD (ng mL ⁻¹)	18.8	9.4	0.5	-
	Equation	y = 0.72x + 0.34 (R ² = 97)*	y = 0.80x + 0.65 (R ² = 0.99)*	y = 0.59x + 1.58 (R ² = 0.99)*	-
PSA spiked in 75% human serum	LOD (ng mL ⁻¹)	-	18.8	2.3	0.29
	Equation	-	y = 1.04x + 0.03 (R ² = 0.98)* CV = 5 to 14 %	y = 0.73x + 1.00 (R ² = 0.99)* CV = 7 to 22 %	y = 1,391.12ln(x) + 1,173.16 (R ² = 0.96) CV = 1 to 8 %

* y = log (response) x = log (concentration)

Capacitive biosensor development for PSA detection:

- A new interdigitated capacitive electrode array and PCB were designed and fabricated to perform PSA detection. Antigen binding assays were developed

to capture the PSA molecule in buffer solutions achieving a detection limit of 15.6 ng mL^{-1} with a linear dynamic detection range of $15.6 - 250 \text{ ng mL}^{-1}$ PSA.

CHAPTER VI: FUTURE WORK

6 FUTURE WORK

The overall results achieved from the study include the development of fabrication protocols for different sensor surface chemistries and this included a novel sensor surface fabrication. Additionally PSA immunoassay was performed successfully using biosensors to detect PSA not only in buffer but also in patient serum samples at clinical levels. However, there are several suggestions to improve the developed surfaces or immunosensors for the future applications:

Sensor surface chemistry development:

- The developed novel polymer surface may be optimised further and more experimental work is needed to convert the application to a full patent.
- CMD surface fabrication protocol needs to be optimised further to enable assays using SPR2 biosensors.

Development of a QCM and SPR assay for PSA detection:

- New biomarkers may be added to obtain a multi-analyte test for prostate cancer to increase the predictivity of the test and antibodies that do perform well for biosensors need to be chosen for the immunoassays of potential biomarkers.
- Sample handling need to be optimised to perform assays on patient serum samples.

Capacitive biosensor development for PSA detection:

- The developed prototype for capacitive biosensor may be improved that include fluidics and automated signal processing.

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APPENDIX A- PUBLICATIONS

Patent application

Y. Uludag and **I. Tothill**, “High capacity polymer surface for immobilisation of biomolecules”, patent application date lodged: 10 July 2009, GB0911634.4.

Published application note

Detection of Total Prostate Specific Antigen in Serum, Sierra Sensors GmbH, Hamburg/Germany (www.sierrasensors.com)

Papers

- A. **Y. Uludag**, **I.E. Tothill**, “Development of a Sensitive Detection method for Cancer Biomarkers in Human Serum (75%) using a Quartz Crystal Microbalance Sensor and Nanoparticles amplification system”, *Talanta*, Vol 82(1), 277-282
- B. **A. Bossi**, **M.J. Whitcombe**, **Y. Uludag**, **S. Fowler**, **I. Chianella**, **S. Subrahmanyam**, **I. Sanchez**, and **S.A. Piletsky**, “Synthesis of controlled polymeric cross-linked coatings via iniferter polymerisation in the presence of tetraethyl thiuram disulphide chain terminator”, *Biosensors Bioelectronics*, Vol 25 (9), 2149-2155, 2010.
- C. **Y. Uludag**, **I.E. Tothill**, “Picomolar detection of total PSA in human serum (75%) using nanoparticles with an SPR biosensor and comparison of the results with QCM biosensor”, ready for submission.
- D. **Z. Altintas**, **Y. Uludag**, **I. E. Tothill**, “Development of the immobilization surfaces in SPR biosensor for the detection of protein and DNA molecules “, ready for submission.

Conference poster and oral presentations

- A. **Y. Uludag**, I.E. Tothill, “Highly sensitive cancer biomarker detection in human serum (75%) using real-time label-free biosensors”, poster presentation, Biosensors 2010 (Glasgow, UK) conference, 26-27.05.2010.
- B. **Y. Uludag**, S.S. Kallemudi, Z. Altintas, I.E. Tothill, Y. Gurbuz, “Novel label-free capacitive sensor based on interdigitated electrode arrays for multiplex point-of-care testing”, poster presentation, Biosensors 2010 (Glasgow, UK) conference, 26-27.05.2010.
- C. **Y. Uludag**, I.E. Tothill, “Point of Care Testing for Prostate Cancer Diagnosis” poster presentation, Molecular Diagnostics Europe Conference, 19-20 May 2009, Stockholm, Sweden.
- D. **Y. Uludag**, E. Nna, T. Baker, I.E. Tothill, “Point-of-care testing for prostate cancer diagnosis”, Poster presentation, Advances in Biosensors for Point of Care Applications Conference, 25 February 2009, NPL, London, UK.
- E. **Y. Uludag**, I.E. Tothill, “Prostate Cancer Diagnosis Using an Affinity Sensor” oral presentation, Multistrand Conference, 6-7 May 2008, Cranfield University, UK.

APPLICATION NOTE 102

Detection of Total Prostate Specific Antigen in Serum

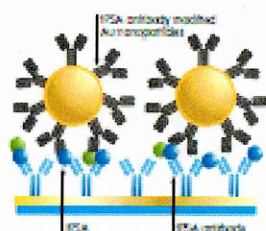
Employing the QCMA-1 Molecular Analysis System in Point-of-Care testing for prostate cancer diagnosis

Based on the work of Yildiz Uludag and Ibtisam E. Tothill*

Cranfield Health, Cranfield University, Cranfield, Bedfordshire, MK43 0AL, England, United Kingdom.

Introduction

Prostate cancer is the most common form of cancer for European men, with 301,500 cases reported annually¹. Early detection is critical as there is no cure once it spreads to other organs. Increased levels of prostate specific antigen (PSA) is a primary indicator of prostate malignancy, making it an ideal biomarker for cancer diagnosis^{2,3}. As PSA testing is typically performed in centralized laboratories using slow and expensive procedures⁴, there is a need to develop a fast, simple and cost effective detection methods.

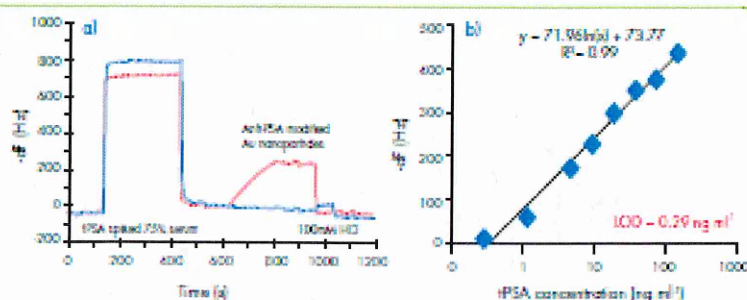


Schematic of sensor surface showing immobilized anti-PSA antibodies, bound PSA and secondary anti-PSA gold nanoparticle conjugated antibody.

Methodology

The QCMA-1 Analysis System from Sierra Sensors GmbH was employed to detect and characterize total PSA (tPSA; free and complex PSA) in serum samples. Briefly: Anti-PSA and a control antibody were linked to the two different detection spots of a QCMA Affinity Sensor. Concentrations of tPSA in serum were then passed over both sensors and the binding measured. The affinity (KD) of tPSA binding to anti-PSA was characterized from this direct interaction. To improve detection sensitivity for the concentration analysis of tPSA in 75% serum, anti-PSA antibody conjugated to gold nanoparticles was used as a signal amplification step in a sandwich assay format. These assays were also run on an SPR based biosensor (Biacore 3000, GE Healthcare) for comparative purposes.

a) QCMA-1 sensogram of tPSA analysis showing binding response of nanoparticle conjugated antibody to bound tPSA, b) Calibration curve of tPSA concentration in 75% serum samples

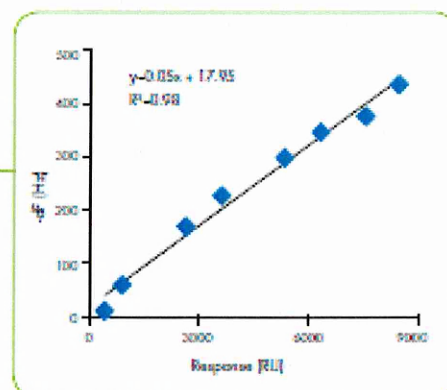


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Biosensor Technologies

APPLICATION NOTE 102

Comparison of QCMA-1 and Biacore 3000 assay results for tPSA concentration analysis in 75% human serum



Results

The KD for the interaction of free PSA binding to anti-PSA determined using the QCMA-1 was 5.56×10^{-11} M [Biacore = 9.46×10^{-11} M]⁴. The limit of detection for tPSA in 75% human serum using the QCMA-1 was 0.29 ng/ml, with a linear detection range of 0.29 – 150 ng/ml (same for Biacore 3000). The tPSA concentration of male human serum test sample (from Sigma) was determined to be 0.41 ng/ml using QCMA-1 (0.56 ng/ml for Biacore 3000).

Conclusions

The interaction of Prostate Specific Antigen and anti-PSA antibodies were investigated using the QCMA-1. The results of this study indicate that QCMA-1 has the potential to be employed as a fast, accurate, and simple detector of tPSA in patient samples. The investigation also demonstrates the performance of QCMA-1 compares well to that of the Biacore 3000 system.

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Development of a sensitive detection method of cancer biomarkers in human serum (75%) using a quartz crystal microbalance sensor and nanoparticles amplification system

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ABSTRACT

A simple and sensitive sensor method for cancer biomarkers (prostate specific antigen (PSA) and PSA- α 1-antichymotrypsin (ACT) complex) analysis was developed, to be applied directly with human serum (75%) by using antibody modified quartz crystal microbalance sensor and nanoparticles amplification system. A QCM sensor chip consisting of two sensing array enabling the measurement of an active and control binding events simultaneously on the sensor surface was used in this work. The performance of the assay and the sensor was first optimised and characterised in pure buffer conditions before applying to serum samples. Extensive interference to the QCM signal was observed upon the analysis of serum. Different buffer systems were then formulated and tested for the reduction of the non-specific binding of sera proteins on the sensor surface. A PBS buffer containing 300 $\mu\text{g mL}^{-1}$ BSA, 0.5 M NaCl, 500 $\mu\text{g mL}^{-1}$ dextran and 0.5% Tween 20, was then selected which eliminated the interfering signal by 98% and enabled the biomarker detection assay to be performed in 75% human serum. By using Au nanoparticles to enhance the QCM sensor signal, a limit of detection of 0.20 ng mL^{-1} PSA and PSA-ACT complex (in 75% serum) with a linear dynamic detection range up to 150 ng mL^{-1} was obtained. With the achieved detection limit in serum samples, the developed QCM assay shows a promising technology for cancer biomarker analysis in patient samples.

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1. Introduction

The World Health Organisation (WHO) states that around 13% of all deaths worldwide in 2007 were caused by cancer [1]. Early and accurate detection of cancer is very important before it spreads out to the other organs of the body and this makes early diagnosis very important for successful treatment of the disease. The detection of cancer biomarkers in patient samples provides an effective way to diagnose and treat the disease. Recent advances in the area of sensor technology and microarrays have enabled the miniaturisation of the devices and multiplex testing of a range of analytes. Therefore, biosensor technology has the potential to produce point of care cancer testing devices that detects biomarkers [2].

Among different cancer types, prostate cancer which is a complex and multifactorial disease is the commonest form of cancer in men in Europe (301,500 incident cases, 24.1% of all cancer cases) [3]. The increase in prostate specific antigen (PSA) levels in serum

above the normal limits is the primary indication of prostate malignancy; therefore PSA is used as a biomarker for the diagnosis and prognosis of the prostate cancer [4,5]. Several biosensor systems have been applied in the past for the detection of PSA: electrochemical [6–8], optical [9–11], fluorescence/chemiluminescence [12,13], microcantilever [14,15] and quartz crystal microbalance (QCM) biosensors [16,17] were used for the detection. The detection limit of PSA using these systems varies between 0.2 $\mu\text{g mL}^{-1}$ and 10 ng mL^{-1} . The detection signal has been amplified by means of a sandwich assay with Au nanoparticles or the use of carbon nanotubes. In most cases biomarker detection test was performed only in buffered pure solutions rather than serum. To obtain clinically relevant results, it is essential to perform the biomarker test in human serum. The main difficulty of using serum as the assay media is high non-specific interaction between the sensor surface and serum proteins. A number of strategies have been employed to reduce the non-specific binding of clinical samples. As an example Cao et al. used mixed self-assembled monolayer coated surface which contains ethylene glycol units [9]. Kurosawa et al. used 2-methacryloyloxyethyl phosphorylcholine polymer as blocking agent [18] and Situ et al. employed additives in the buffer to lower the non-specific binding of serum proteins [19] to the sensor surface. The above described methods can either be applied

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individually or together to reduce the non-specific binding of serum proteins achieving different success.

In this paper we describe the development of a simple and rapid detection method for cancer biomarkers analysis using a quartz crystal microbalance (QCM) biosensor. A QCM consists of a thin quartz wafer sandwiched between a pair of electrodes. The mode of oscillation depends on the cut and geometry of the quartz crystal. Mass addition or loss on the sensor surface results in frequency change and hence measuring molecular interactions that occur on the sensor surface without the need of a label. By employing Au nanoparticles in the assay design, it is possible to increase the mass due to antigen binding and further enhance the sensitivity of the assay [20,21]. However, the frequency is also affected by viscosity change of the assay media and charge of the interaction molecules [22]. Therefore, care needs to be taken to subtract the controls and extract the real response due to specific molecular interactions. The sensitivity enhancement using nanoparticles has been usually achieved by modifying a secondary antibody to the nanoparticles and performing a sandwich assay.

In this work an investigation was performed to develop and optimise an immunoassay to detect biomarkers in human serum on the QCM sensor chip. To minimise the matrix effect of human serum, the addition of detergent, salt and other additives to the buffer solution was investigated. PSA and PSA- α ACT complex were used as the cancer biomarkers for detection and an immunoassay was developed and performed in buffer and in human serum.

2. Materials and methods

PSA, monoclonal PSA detection (cat no: MCA2561) and capture antibodies (cat no: MCA2560) were obtained from AbD Serotec (Kidlington, UK). ACT-PSA complex was purchased from BioPacifica (CA, USA). Mouse IgG (cat no: 015-000-003) usually used as a control antibody was obtained from Stratech Scientific Ltd. (Jackson ImmunoResearch (Newmarket, UK). Human serum and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (Poole, UK). All other chemicals were of analytical grade.

2.1. Instrumentation

A fully automated QCMA-1 biosensor instrument and sensor chips were obtained from Sierra Sensors GmbH (Hamburg, Germany). Au coated QCMA-1 sensor chips (20 MHz) possess two sensing arrays each, enabling the measurement of active and control sensor surfaces simultaneously (Fig. 1S). The operating temperature of the assays was 25 °C and the flow rate of the buffer was 80 μ L min⁻¹ throughout the assay. The data presented in this work are the averages of 4 data points for the assays described unless otherwise stated.

2.2. Modification of Au nanoparticles with anti-PSA detection antibody

PSA detection antibody was added to the 40 nm Au nanoparticle solution and incubated at room temperature on a shaker. BSA was added to the solution to ensure that the Au nanoparticles are all coated with antibody or protein. After spinning 20 min at 4 °C, the antibody modified nanoparticles were recovered and re-suspended in PBS/T buffer. The concentration of nanoparticles was determined by a spectrophotometer at 520 nm wavelength. The antibody modified Au nanoparticles were then stored in the fridge (4 °C) until use.

2.3. Sensor surface modification

Initially gold coated QCMA-1 sensor chips were coated with self-assembled monolayer (SAM) by immersing the sensors in 2 mM

ethanol solution of mercaptoundecanoic acid overnight followed by rinsing with ethanol and water and then dried under nitrogen. The sensors were stored at 4 °C till use. For the AFM (Atomic Force Microscopy) study, Dimension 3000 SPM instrument (Veeco Instruments Ltd., Cambridge) was used with silicon probes (type: PPP-NCH-50). The analysis was carried out at tapping mode. AFM images of QCMA-1 sensor were taken before and after surface chemistry application. For the assay, the SAM coated sensor chip was first docked to the instrument and primed with running buffer (10 mM PBS, pH 7.4) at a flow rate of 80 μ L min⁻¹ until use. Monoclonal anti-PSA antibodies (capture antibody) and Mouse IgG antibody (control antibody) were then immobilised on the sample and control sensing arrays respectively using conventional amine coupling chemistry. Sensor surfaces were first activated with a 1:1 mixture of 400 mM EDC and 100 mM NHS by injecting simultaneously across the two sensing spots for 3 min (240 μ L). A 15–30 or 50 μ g mL⁻¹ anti-PSA antibody (in sodium acetate buffer, 10 mM, pH 5.5) was injected on the active sensor array and 15–30 or 50 μ g mL⁻¹ mouse IgG (in sodium acetate buffer, pH 5.5) was injected on the control sensor array for 3 min (240 μ L). The surfaces were then blocked with 50 μ g mL⁻¹ BSA in PBS for 3 min (240 μ L). Non-reacted NHS esters were capped with 1 M ethanolamine, pH 8.5 for 3 min (240 μ L). The frequency changes were recorded 2 min after the protein injection was completed and the signal reached steady state. The running buffer was changed to PBS containing 0.005% Tween (PBS/T) for the binding assay studies.

2.4. Detection of PSA

In serum, PSA is found either in free form or as a complex with alpha 1-antichymotrypsin (ACT) (ACT-PSA, MW 96 kDa). Total PSA (tPSA) refers to the PSA in both forms (PSA and ACT-PSA complex). In the current study to prepare tPSA 1 to 1 mixture of PSA and ACT-PSA was used. PSA or tPSA was diluted at specified concentrations (0.29–5000 ng mL⁻¹) in PBS/T buffer containing 5 μ g mL⁻¹ BSA or 10–75% human serum in PBS/T buffer containing additives. These solutions were then injected over the PSA capture antibody and mouse IgG immobilised surfaces for 3 or 5 min to allow binding interactions (240 or 400 μ L). The frequency changes due to PSA/tPSA binding were recorded at 180 s after the injection started. After the binding of PSA/tPSA either surface was regenerated by injection of 100 mM HCl (1 min, 80 μ L) or the assay was continued to perform a sandwich assay.

After the binding of PSA/tPSA to the sensor surface, 1.5 μ g mL⁻¹ PSA detection antibodies or PSA detection antibody modified Au nanoparticles were injected on the sensor surface for 3 or 5 min (240 or 400 μ L). After 3 min dissociation period under running buffer flow, surfaces were regenerated by injection of 100 mM HCl (1 min, 80 μ L) (and additional 20 mM NaOH (1 min, 80 μ L) was injected if PSA spiked human serum was used). The frequency changes due to PSA detection antibody binding were recorded 3 min after the injection started. The limit of detection (LOD) was calculated as the signal obtained from the PSA concentration that is equivalent to the 3 times the standard deviation of the signals obtained from the blank standards.

3. Results and discussion

3.1. Optimisation of sensor signal

Bare gold QCMA-1 sensor chips were employed in this work as the sensor platform for PSA detection. Each chip consists of two sensing array for active and control sample testing. The modification of the chips using self-assembled monolayer (SAM) was carried out on the sensor surface. Atomic force microscopy (AFM) images of

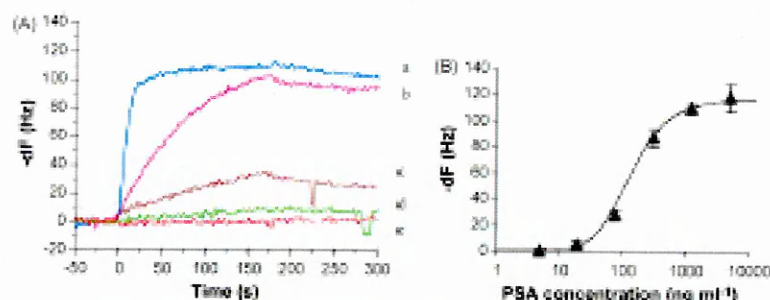


Fig. 1. (A) QCM sensor response for different concentrations of PSA samples (3 min injection). After each sample injection, the surface was regenerated with a 1 min injection of 100 mM HCl (data not shown). (a) 5000 ng mL⁻¹, (b) 312 ng mL⁻¹, (c) 17 ng mL⁻¹, (d) 8.8 ng mL⁻¹, (e) all control subtracted data and (e) the response obtained from the binding of 5000 ng mL⁻¹ PSA to mouse IgG immobilised control surface. (B) The frequency response of PSA binding to the immobilised PSA antibody versus (log) PSA concentration plot.

a sensor chip were taken before and after the SAM coating (Fig. 2S). The results indicate that SAM modification caused a change of the root mean square (rms) roughness from 5.5 to 3.1 nm due to surface coating. The SAM coated sensor chip was first dunked to the QCM-A1 instrument and primed with running buffer to wet the sensor chip and continues buffer flow (80 μ L min⁻¹) was started. Anti-PSA capture antibody and mouse IgG were then immobilised on the active sensor array and the control sensor array respectively using conventional EDC-NHS chemistry [23]. A 3 min injection of antibodies was sufficient to achieve signal saturation; therefore, the immobilisation time was kept at 3 min for the assay. Three different concentrations were used for antibody immobilisation and from these 30 μ g mL⁻¹ was chosen as the optimum concentration to immobilise the antibodies. Anti-PSA capture antibody immobilised sensors produced an average frequency change of 380 ± 38 Hz and mouse IgG immobilised sensors produced a frequency change of 520 ± 15 Hz (Fig. 3S).

The PSA non-specific binding (diluted in PBS/T buffer containing 5 μ g mL⁻¹ BSA) to the control sensor array was then examined by conducting binding tests. The results show that the control sensor surface did not give any frequency change even if the highest concentration of PSA (5 μ g mL⁻¹) was used (Fig. 1, trace e). To examine the non-specific binding of BSA on the PSA capture antibody immobilised surface, BSA (5 μ g mL⁻¹) was injected for 3 min and the non-specific binding of BSA to the surface was detected as 4 ± 1 Hz ($n = 3$, data not shown).

For the sandwich assay procedure, anti-PSA detection antibody (3 μ g mL⁻¹) was injected in the absence of the antigen (PSA) on to the PSA capture antibody immobilised surface and the non-specific binding was detected as 5 ± 2 Hz ($n = 3$, data not shown). The non-specific responses were subtracted from the PSA or PSA detection antibody binding data.

3.2. Determination of kinetic constants for PSA detection assay

The calibration curve obtained with PSA binding (3 min) to the PSA capture antibody immobilised surface in a concentration range between 4.7 and 5000 ng mL⁻¹ is shown in Fig. 1 and Fig. 4S. PSA binding response curves were then fitted to 1:1 Langmuir binding model to determine the binding association and dissociation rates [24], from which K_D value was calculated as 5.56×10^{-10} M and R_{max} as 108 Hz (Table 1). Karlsson et al. calculated the affinity of PSA antibody towards PSA as 3.3×10^{-9} M (antibody from Fitzgerald Industries Int., clone M212091) [25]. Katsamha et al. calculated the affinity as 1.1×10^{-9} M (antibody from Fitzgerald Industries Int., clone M612166) [26].

To enhance the sensor signal and improve the sensitivity of the method, a sandwich assay approach was followed employing PSA detection antibody. The calibration curve was obtained for the sandwich assay in a concentration range between 150 and 2.3 ng mL⁻¹ that is relevant for prostate cancer diagnosis (Fig. 4S, trace h). This assay resulted in 4.7 ng mL⁻¹ PSA as detection limit which is four folds more sensitive than the direct assay (Table 2). The experiments have shown that the chosen anti-PSA antibody has good affinity for a successful immunoassay and the sandwich assay resulted in a clinically relevant detection limit. Consequently experiments were performed to optimise the assay so that biomarker detection could be performed in human serum.

3.3. Buffer optimisation for human serum sample analysis

There is a significant difference in the limit of detection when buffer or clinical sample (serum) is used as the assay media. For example, although Cao et al. performed the PSA-ACT detection assay using only 10% human serum; the limit of detection has changed from 10.2 ng mL⁻¹ (in buffer) to 18.3 ng mL⁻¹ (in 10% serum) [9]. This change in the detection limit, when clinical samples are used, is mainly due to lower signal to noise ratio resulting from the high non-specific binding of sera proteins/antibodies to the sensor surface. This is especially noticeable when label-free biosensors are employed, and this prevents researchers from the use of high concentrations of human serum. Therefore, most analyses are conducted using concentration of human serum in the range 10–50% [18,19,27].

In the current study we investigated the use of additives to minimise the adsorption of sera proteins to the sensor surface. Initially, a 10% human serum diluted in PBS/T buffer was injected on the mouse IgG immobilised sensor surface. A 1400 Hz response was obtained from this solution and after buffer flow started, the response was reduced to 1370 Hz (Fig. 2, A-trace 1). From the results it was evident that human serum proteins caused a significant non-specific binding on the surface. To reduce this non-specific binding salt, BSA, dextran and Tween 20 were added to the buffer at varying concentrations. Increase the salt concentration increases the ionic strength of the solution and that lowers the electrostatic attraction of human serum proteins to the sensor surface. Therefore, high salt

Table 1
Results of kinetic calculations for PSA direct assay.

K_D	5.56×10^{-10} M
k_a	1.23×10^{10} M ⁻¹ s ⁻¹
k_d	6.83×10^{-14} s ⁻¹
R_{max}	108 Hz

Table 3

The summary of the PSA detection assay results.

	Direct assay in PBS/T	Sandwich assay in PBS/T	Sandwich assay in 75% serum	Sandwich assay in 75% serum using Au nanoparticles
LOD	0.6 ng mL ⁻¹	4.7 ng mL ⁻¹	9.4 ng mL ⁻¹	0.29 ng mL ⁻¹
Linear range	0.6–150 ng mL ⁻¹	4.7–150 ng mL ⁻¹	9.4–150 ng mL ⁻¹	0.29–150 ng mL ⁻¹
Equation	$y = 0.07x + 0.27$ ($R^2 = 1.00$) ^a	$y = 0.01x + 0.35$ ($R^2 = 0.99$) ^a	$y = 0.006x + 0.33$ ($R^2 = 0.98$) ^a	$y = 71.95 \ln(x) + 71.77$ ($R^2 = 0.99$)

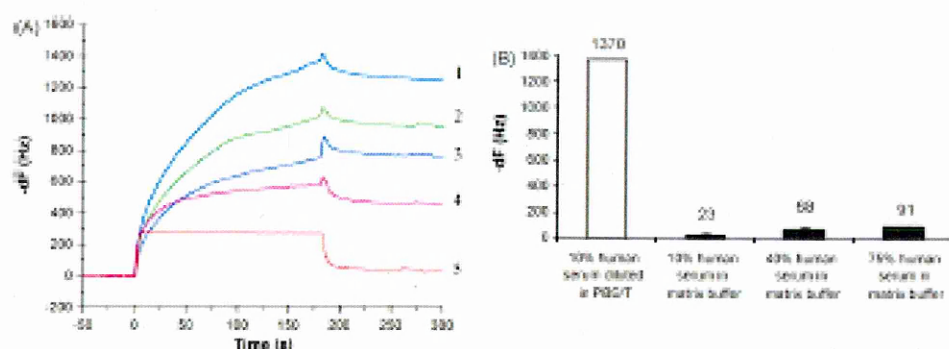
^a $y = \log(\text{response})$; $x = \log(\text{concentration})$.

Fig. 2. (A) Effect of changing the buffer composition on the human serum signal. The injection of 100 human serum in PBS/T buffer (1) and PBS/T buffers containing additives at varying concentrations (buffer 2 (100 $\mu\text{g mL}^{-1}$ BSA), 3 (200 $\mu\text{g mL}^{-1}$ BSA), 4 (200 $\mu\text{g mL}^{-1}$ BSA and 0.5% Tween), 5 (200 $\mu\text{g mL}^{-1}$ BSA, 0.5 M salt, 500 $\mu\text{g mL}^{-1}$ dextran and 0.5% Tween 20)) to mouse IgG immobilised sensor chips. (B) Effect of increasing human serum concentration on the sensor signal. Human serum was diluted in matrix buffer (buffer 5; contents described above) at varying concentrations and injected (3 min) on mouse IgG immobilised surface.

concentration was used to reduce proteins adsorption to the sensor surface. Dextran and BSA were used to help in preventing serum protein absorption. It is common to employ detergents to lower the non-specific protein binding; therefore the concentration of Tween 20 was increased in the solution [28]. These additives at varying concentrations were added to prepare 10% human serum and injected over the mouse IgG immobilised sensor surface for 3 min (Fig. 2A). The lowest non-specific binding was observed when the serum is diluted in additives containing PBS/T buffer that had final concentrations of 200 $\mu\text{g mL}^{-1}$ BSA, 0.5 M salt, 500 $\mu\text{g mL}^{-1}$ dextran and 0.5% Tween 20 in solution. The response 180 s after the injection of 10% human serum diluted in this matrix elimination buffer (matrix buffer) was found as 251 ± 18 Hz ($n = 3$) and after the buffer flow started the response was reduced to 23 ± 12 Hz ($n = 3$). As it can be seen from Fig. 2, A-trace 1, the injection of human serum in PBS/T buffer resulted in a trace that showed a curved structure indicating the binding of sera proteins to the sensor surface and there was little dissociation after the injection stopped and

buffer flow was started. Whereas when human serum diluted in the matrix buffer was injected (Fig. 2, A-trace 5), the response trace showed an instant sharp increase up to ca. 250 Hz and remained showing no curvature indicated that the bulk of this response was due to the use of additives and therefore this matrix buffer was then used for all further assays using human serum. Fig. 2B shows the comparison of non-specific binding results for 10%, 40% and 75% human serum diluted in the matrix buffer developed in this work and injected to mouse IgG immobilised surface. The low non-specific binding values for these three concentrations of human serum (highest 91 $\pm$ 10 Hz for 75% human serum) indicated that the matrix buffer was effective even for high serum concentrations. For all the forthcoming experiments human serum was always diluted with the matrix buffer.

These results showed that by applying the matrix buffer in the assay method, a 98% reduction in non-specific binding of human sera proteins was achieved. If this result is compared to other recently published work: Snu et al. achieved 94% reduction in non-

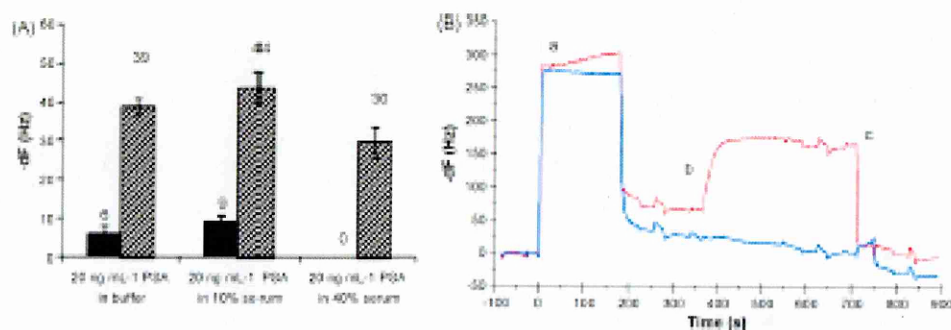


Fig. 3. (A) Direct (black) and sandwich (striped) PSA assay in PBS/T buffer, 10% human serum and 40% human serum diluted in matrix buffer ($n = 3$). (B) Direct and sandwich assay with 70 ng mL⁻¹ PSA spiked 10% human serum on active and control sensing spots. Injections of (a) 70 ng mL⁻¹ PSA spiked 75% human (b) 1.5 $\mu\text{g mL}^{-1}$ anti-PSA antibody (c) 100 mM NaCl.

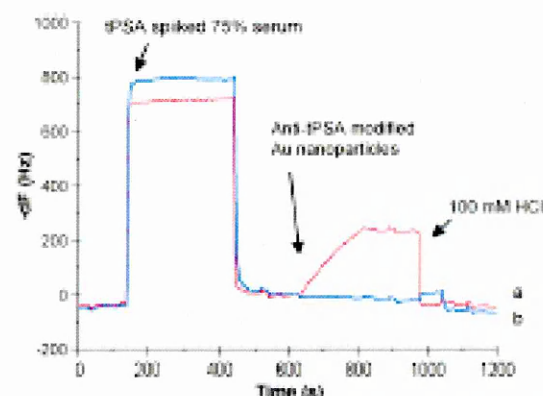


Fig. 4. Injections of 9.4 ng mL⁻¹ tPSA spiked serum and tPSA antibody modified Au nanoparticle on active (a) and control (b) surfaces.

specific binding by using CM5 (carboxymethyl dextran) surface and a formulated buffer together and Trevino et al. achieved 88% reduction in non-specific binding of serum by using another formulated buffer [19,29].

The PSA assay was then performed using 20 ng mL⁻¹ PSA spiked in 10% and 40% human serum. When the results were compared with PSA assay in PBS/T buffer, it was seen that both direct and sandwich PSA assay results in buffer or spiked in 10% human serum (diluted in matrix buffer) were very close to each other and within the standard deviation (Fig. 3A). However, as the human serum concentration increased to 40%, the response from direct assay was eliminated but still there was a response from the injection of PSA detection antibody (sandwich assay). Although this result was lower than the assay in PBS/T buffer (or assay in 10% human serum) still good enough to perform the assay in high human serum concentrations. An example to direct and sandwich assay response of PSA spiked in 10% human serum is shown in Fig. 3B.

3.4. Detection of tPSA in 75% human serum

A calibration curve was obtained for the sandwich assay in a concentration range between 2.3 and 150 ng mL⁻¹ tPSA spiked in 75% human serum. The non-specific binding of PSA detection antibody after the injection of 75% human serum (without spiked PSA) was 2 ± 1 Hz, if assay performed on mouse IgG immobilised surface and 10 ± 3 Hz if assay performed on PSA capture antibody immobilised surface. The PSA detection antibody binding responses were obtained after the subtraction of the non-specific binding response. The detection limit obtained for this assay was 9.4 ng mL⁻¹ (Fig. 5-trace a). However this result was not sensitive enough for PSA detection at the required levels in serum. To enhance the sensitivity further 40 nm Au nanoparticles were modified with PSA detection antibody and sandwich assay was performed to detect tPSA. The use of Au nanoparticles lowered the detection limit down to 0.29 ng mL⁻¹ (Fig. 5-trace b), which is well below the required limit of detection. Table 2 summarises the results of the assays in buffer and in human serum.

The specificity of the tPSA interaction with the sensor surface was tested by employing a mouse IgG surface on the control sensor. The non-specific binding of PSA detection antibody modified Au nanoparticles after the injection of 75% human serum (with or without spiked tPSA) resulted in no response on mouse IgG immobilised surface (Fig. 4-trace b). The binding of PSA detection antibody mod-

ified Au nanoparticles after the injection of 75% human serum (without spiked tPSA) resulted in 0 ± 2 Hz response on anti-PSA capture antibody immobilised surface, since non-specific binding on to MlgG surface was very low, this result was purely due to the tPSA content of the serum obtained from Sigma. While creating the calibration curve, this response was deducted from the responses obtained to get the binding results due to the spiked tPSA. Later after calibration curve of tPSA assay was obtained, it was calculated that serum from Sigma used for the study contains 0.41 ng mL⁻¹ tPSA (Fig. 5).

In conclusion, the limit of detection of the biomarker assay was 0.29 ng mL⁻¹ (corresponds to 0.39 ng mL⁻¹ in 100% serum) with a linear detection range of 0.29–150 ng mL⁻¹ and the coefficient of variation (CV) laid between 1% and 9% across the range. These results show that the assay developed with the formulated matrix buffer and Au nanoparticle signal amplification, have potential for the rapid and sensitive detection of serum biomarkers in patient samples.

The number of publications related to the detection of PSA using QCM biosensor is very limited and this indicates that application of QCM biosensors to PSA detection is a newly developing area. An example to a previous study for PSA assay using QCM biosensor has been the study performed by Zhang et al. which involve the use of an insert-plug model of piezoelectric immunosensor. After 40 min of reaction at 37 °C in a static cell with a 10 MHz quartz crystal, Zhang et al. achieved linear detection of 1.5–40 ng mL⁻¹ PSA in 100% serum [17]. Although the detection limit obtained from this study was low enough, the reaction time was long and assay needs to be performed at elevated temperatures. Additionally the dynamic range of the assay was limited. In another study, Ding et al. used yeast cell modified QCM sensors to immobilise PSA antibodies [16]. The binding of PSA to the PSA antibody immobilised sensor was recorded after the binding reached equilibrium using a 9 MHz quartz crystal and magnetic stirrer agitated static cell. With this method Ding et al. achieved detection of PSA in the range of 5–604 ng mL⁻¹ in 100% serum. Both applications mentioned above were performed using non-automated equipment that due to their manual operation may not only cause variations between the assays but also are not suitable for clinicians use. In another study Cao et al. performed 10 min PSA-ACt assay in 10% human serum using an automated SPR biosensor and obtained a detection limit of 18.1 ng mL⁻¹ that corresponds to LOD of 181 ng mL⁻¹ for the whole serum [8].

In our study although 75% serum was used as assay matrix, the detection limit obtained was 0.29 ng mL⁻¹ for a 8 min assay which corresponds to 0.39 ng mL⁻¹ tPSA in the whole serum indicating the good performance of the optimised assay. Additionally, although high concentration of serum was used for the assay, matrix buffer limited the non-specific binding of the proteins on to sensor spots and allowed complete regeneration of the sensor surface by short injections of 100 mM HCl and 20 mM NaOH. This enables the repeated use of the same sensor chip for several times to analyse multiple serum samples to detect tPSA.

The experimental conditions reported in the literature vary between the assays significantly such as, assays in static or in flow, temperature, assay time and the antibody used against PSA. All these contribute to the assay sensitivity and hence make it difficult to compare the results effectively. When compared briefly to similar label-free PSA assays such as above, the assay developed in this study, with total 8 min reaction time, repeated usability and LOD of 0.29 ng mL⁻¹ in 75% human serum, proves the advantage of the optimised assay format suitable for point of care system. Additionally due to it is being rapid and wide detection range the QCM assay developed outperforms the commercial ELISA kits available for biomarker detection (Table 3).

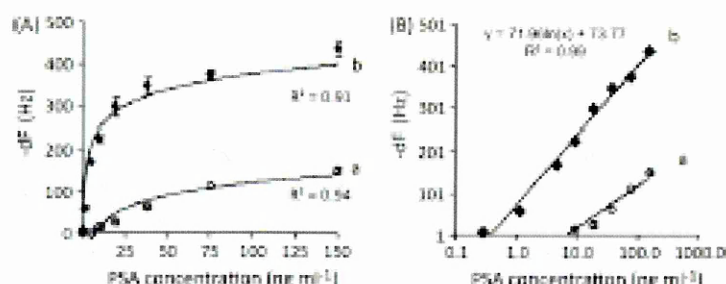


Fig. 5. tPSA at varying concentrations (in 75% human serum) were injected to tPSA capture antibody immobilised QCM sensor surface. Later, in a sandwich assay tPSA detection antibody (a) or antibody modified Au nanoparticles (b) were injected and the frequency changes due to binding of nanoparticles were recorded and calibration curves (A – linear scale; B – log scale) were obtained.

Table 2

The summary of the PSA detection limit and detection ranges for some of the commercial ELISA kits and the developed QCM assay.

	Detection range (ng mL^{-1})	Limit of detection (ng mL^{-1})
The developed QCM assay ^a	0.29–200	0.29
Abcam PSA ELISA kit	1–50	1
MP Biomedicals PSA ELISA kit	2–120	1
Alpco Diagnostics PSA ELISA kit	2–120	1
Calbiotech PSA ELISA kit	0.585–50	0.585
Alpha Diagnostic PSA ELISA kit	0.2–60	0.20

^a Both detection range and limit is given based on 100% serum.

4. Conclusion

Early diagnosis of cancer biomarkers is essential for successful treatment of the disease. The current biomarker tests are ELISA type and usually performed at centralised labs using automated devices. In this work, a groundwork study was described for the use of QCM biosensor to perform cancer biomarker detection test. A new buffer was formulated to eliminate 98% of the non-specific human serum protein binding to the sensor surface. A sandwich assay using QCM-A1 affinity sensor chips resulted in detection of tPSA concentrations as low as 0.29 ng mL^{-1} in 75% human serum. Short assay time, repeated usability of the same sensor chip, ability to detect PSA in high serum concentrations and utilisation of a label-free detection method enables the optimised assay format a promising tool for clinical diagnosis/prognosis.

Acknowledgement

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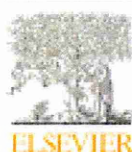
Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2016.04.034.

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Synthesis of controlled polymeric cross-linked coatings via iniferter polymerisation in the presence of tetraethyl thiuram disulphide chain terminator

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ABSTRACT

A "grafting from" approach has been used for controlled deposition of cross-linked polymers by living radical polymerisation. Borosilicate glass was modified with *N,N*-diethylaminodithiocarbamylpropyltrimethoxysilane, in order to confine the iniferter reactive groups solely at its surface, then placed in solution with monomers and cross-linker. The polymerisation was initiated by UV irradiation. Formation of the cross-linked polymers was studied in terms of time course of the reaction, type of monomers incorporated and influence of oxygen. Grafted surfaces were characterised by AFM, FT-IR, ellipsometry and contact angle measurements.

The ability to control the grafted layer improved dramatically when the chain terminator agent, *N,N*-*N,N*-tetraethyl thiuram disulphide (TED) was added. Upon irradiation TED increases the concentration of passive capping radicals and decreases the possibility of recombination of active macro-radicals, thus prolonging their lifetime. In the absence of TED the thickness of produced coatings was below 10 nm. TED added at different concentrations assisted in the formation of grafted layers of 10–130 nm thickness. Iniferter chemistry in the presence of TED can be used for growing nanometre-scale polymer layers on solid supports. It constitutes a robust general platform for controlled grafting and offer a general solution to address the needs of surface derivatisation in sensors technology.

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1. Introduction

Mastering the architecture and properties of materials at the nanometre scale is a fundamental step towards the development of leading edge tools in nanotechnology, with practical economic outcomes. Polymeric materials, in particular acrylic- and methacrylic acid based materials, have long been proven suitable for a broad range of applications: separation, recognition, sensing technologies; and are used in biomedical, environmental, security and food-related areas (Yoshida et al., 2006).

Methacrylates are reasonably well characterised and easy to manipulate. Such materials are polymerised traditionally in solution by means of initiators of radical nature through a chain reaction. Polymerisation is distinguished by: (i) free radical poly-

merisation, when the radical initiator is produced by homolytic cleavage of covalent bonds; (ii) ionic chain polymerisation, when cationic and anionic initiators are produced by heterolytic cleavage of ion pairs and (iii) living radical polymerisation, when radicals can be initiated or deactivated in a reversible way (Jagur-Grodzinski, 2001).

Ionic chain polymerisation is suitable for a restricted group of ionisable monomers. Free radical polymerisation is widely exploited for the preparation of commercial plastics, because it is easy to perform, leads to high yield of materials in reasonably short times, and therefore has gained a central role in polymer science. However, free radical polymerisation leads to a heterogeneous population of macromolecules, typically with a broad distribution of molecular weights and poor control over dimensions (Ward and Peppas, 2000). Among the reasons for the formation of structural heterogeneities in the growing networks, the spontaneous tendency of monomers, cross-linkers and solvent to separate during the polymerisation into uniform clusters in different spatial regions is a major factor (Klosterbuer, 1998; Anseth et al., 1996). Such segregation is defined as spinodal decomposition (Asnaghi et al., 1995), it generates local environments composed of molecules with

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similar properties (hydrophobicity and density) and ultimately results in different microgelation environments in the same polymerisation reaction and thereby gives rise to heterogeneities. On the contrary, homogeneous materials possess very desirable characteristics, e.g. optical properties, conductivity, strength and resistance, etc., as evidenced by a comparative analysis of physico-chemical and functional properties of materials with dielectric spectroscopy (Kannurpatti and Howman, 1998). The important practical need for controlled deposition of the layers of cross-linked polymers originates from the development of polymer-based sensors, especially optical sensors such as SPR or fluorescent devices. A possible way to introduce order in the polymerisation process and therefore to control it at a molecular level is to use controlled/living radical polymerisation. Living polymerisation is characterised by reversible transformation of dormant species into reactive free radicals that act as chain propagators. In living polymerisation the rate of initiation is significantly greater than the polymerisation rate (Ruckert et al., 2002).

Among the various living initiators (initiator, transfer agent, co-initiator), dithiocarbamate esters react by the photochemical cleavage of the C–S bond (Otsu and Yoshida, 1982), leading to the formation of two radicals with very different properties. One, derived from the alkyl group, is highly reactive. It initiates polymerisation, which propagates by addition of unsaturated monomers. The other is a much less reactive (dormant), sulphur-centred radical that does not react with monomers (Lambrines et al., 1998; Kazmaier et al., 1995) and acts uniquely as a recapping moiety for the macro-radical growing chain. Otsu et al. (1995) confirmed the living nature of photoinitiator polymerisations in bulk and in solution and studied the effect of different reaction conditions on the polymerisation rate and the production of ordered polymeric chains. Minimal broadening of the molecular weight distribution was observed when the chain extension process was followed by size exclusion chromatography (SEC), accounting for the formation of homogeneous macromolecules (Turner and Eleyens, 1993). The kinetic behaviour of surface-initiated photopolymerisation is quite different from that carried out in bulk or in solution and typically has a first-order dependence on monomer concentration (de Boer et al., 2008; Sankesh et al., 2005). Non-linear growth of the layers with exposure time indicates that irreversible termination reactions take place in the system, leading to the loss of surface-attached free radicals. Therefore, photoinitiator-mediated surface-initiated photopolymerisation was classified as “pseudo-living” (Sankesh et al., 2005, 2008). An important and diverse range of materials is produced by polymerisations involving a degree of cross-linking. This results in materials with greater toughness and strength which find uses in a wide variety of applications: from dental restorative preparations (Fawcett and Moszner, 2003) to contact lenses and optical devices (Zwiers and Durrant, 1985), cell filtration and selective membranes (Natori and Kurita, 2007; Piletsky et al., 2003). In particular, cross-linking has practical importance in the synthesis of recognition materials based on molecularly imprinted polymers (MIPs) (Piletsky et al., 2000). In this context, initiator chemistry has a distinct advantage, since the mild experimental conditions used in initiation by this method will help to preserve the pre-polymerisation complex, essential for the creation of recognition properties in MIPs.

Recently living initiator photopolymerisation has been used for the development of imprinted multi-layers with predetermined selectivities (Ruckert et al., 2002; Pérez-Moral and Mayes, 2007). The present study is intended to further expand the chemistry basis of this work and provide better control over the thickness of surface-confined cross-linked layers, setting the ground for the establishment of a general platform for surface modification, which will favour the growth of surface-anchored cross-linked polymers and might benefit the polymerisation of MIPs at surfaces.

Here, we used a dithiocarbamate ester-based initiator covalently bound to a support to grow controlled surface-grafted polymers. The ‘grafting from’ approach was employed: the support was first activated in order to attach the initiator groups solely at its surface and to confine polymerisation to the surface. The ability to control the grafted layer was studied under various conditions and upon addition of a chain terminator agent. *N,N,N',N'*-tetraethylthiuram disulphide (TED), a symmetrical molecule made of two passive, dormant radicals (Otsu, 2008). The addition of TED to the monomer mixture is reported to act on the equilibrium between the active macro-radicals and passive radicals and increase the length of the surface growing chains. The overall architecture strategy is summarised in Scheme 1. We investigated the effect of TED on polymer growth, in order to control grafting thickness over the range 5–130 nm. The aim was to find a recipe for the growth of nano-scale polymer layers of controlled thickness. The ability to predictably grow such layers would help in the design of recognition layers compatible with sensor systems. Moreover it would aid in the design of multi-layer architectures, consequently allowing the optimisation of electrical conductivity and improved diffusion of analytes thus enabling the preparation of a new generation of highly sensitive polymer-based sensors.

2. Experimental

2.1. Materials

Sodium diethyl dithiocarbamate trihydrate, chloropropyl trimethoxysilane, anetone and 9-anthracenylmethyl methacrylate were purchased from Sigma-Aldrich (Gillingham, UK). All other chemicals were of analytical grade and were used as received. Ultrapure water (Millipore) was used for analysis. NMR spectra were obtained using a JEOL ECX-400 multinuclear NMR spectrometer. NMR solvents were obtained from Geze Scientific (Chelmsford, UK).

2.2. Synthesis of

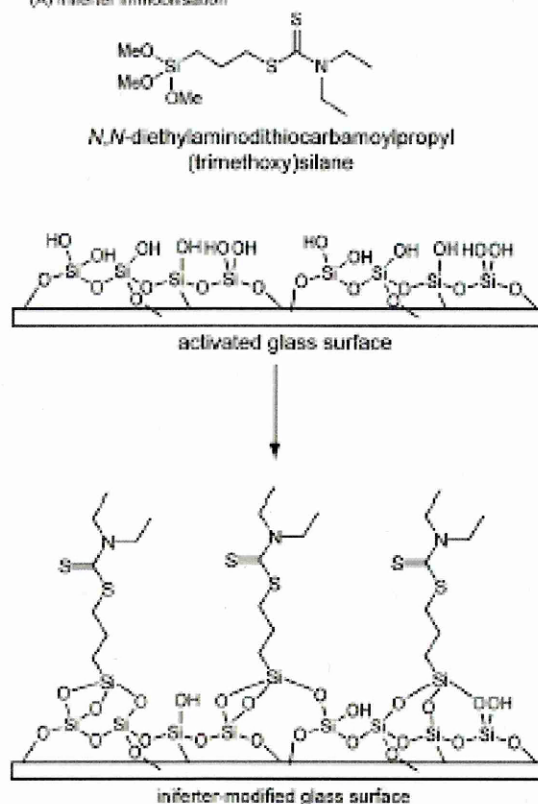
N,N-diethylamino dithiocarbamylpropyl(trimethoxy)silane

The synthesis of the silane living initiator, *N,N*-diethylamino dithiocarbamylpropyl(trimethoxy)silane was prepared by a modification of the procedure of Kobayashi et al. (1992). Diethyl dithiocarbamate trihydrate (3.42 g) was ground to a fine powder in a mortar. The powdered salt was transferred to a flask and the water of crystallisation removed by heating in an oil bath (105 °C), while connected to a vacuum pump for 18 h. Dehydrated dithiocarbamate salt (2.65 g) was suspended in 43 ml of dry acetone in a flask, fitted with a stirrer and condenser, to which was added an amount of chloropropyl trimethoxysilane (2.48 g) to give a 1:2:1 molar ratio of initiator:silane. The mixture was heated at 45–55 °C. Product, in the form of a yellow oil, was formed after 10–15 h. At the end of the reaction, the insoluble salts were removed by filtration, and the solvent evaporated at 40 °C under vacuum. Product formation and purity were confirmed by TLC and NMR. A quantity of 3.89 g of product was formed.

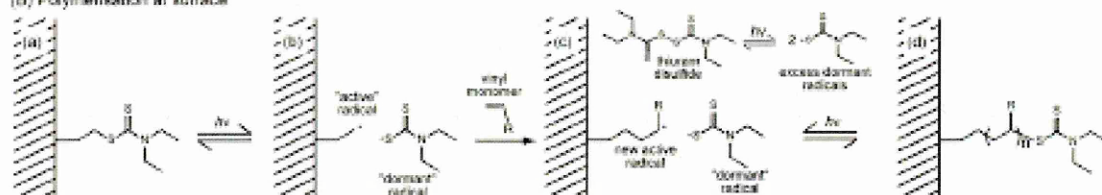
2.3. Activation of borosilicate supports

Support surfaces were borosilicate glass slides cut into rectangles of 0.7 cm × 0.7 cm. Surface activation was achieved by the following steps: 10 min ultrasonication in water, 10 min ultrasonication in 1 M NaOH, 10 min in water. Then, 10 min ultrasonication in 5 M HCl, 10 min ultrasonication in water and 10 min ultrasonication in anetone. Finally surfaces were dried for 2 h in an oven at 80 °C.

(A) Iniferter immobilisation



(B) Polymerisation at surface



2.4. Derivatisation of borosilicate supports with silane bearing iniferter

Activated borosilicate supports were functionalised by immersion for different times (from 1 to 12 h in a 2% (v/v) solution of *N,N*-diethylaminodithiocarbamoylpropyl(trimethoxy)silane in toluene. The supports were then washed in methanol and dried at room temperature. Derivatisation was confirmed by static contact angle measurements.

2.5. Analysis of silane modification by contact angle

Static contact angle (CA) measurements were performed on a Cam 100 optical Angle Meter (KSV Instruments Ltd., Finland). Measurements were repeated four times and the mean value calculated.

2.6. Quantitative estimation of the level of silane modification on the supports

Iniferter-modified borosilicate slides and control slides (not derivatised with the iniferter) were placed in a glass Petri dish filled with 20 ml of a 1% fluorescent monomer, 9-anthracenylmethyl acrylate in toluene, ultrasonicated for 10 min and purged with argon for 20 min. UV irradiation (Philips UV lamp 9 W/cm²) was carried out for 20 and 40 min. After the polymerisation, slides were washed in methanol and dried.

2.7. Polymer grafting

For the grafting, the iniferter-modified supports were placed in a covered Petri-dish and immersed in the polymerisation mixtures:

(P3) 0.5 g MAA, 4.5 g EGDMA, 5 g toluene; (P4) 0.46 g 2-TMAA, 4.29 g EGDMA, 5 g toluene; (P5) 0.665 g EGMP, 4.08 g EGDMA, 5 g toluene. After removal of oxygen by purging with argon, the polymerisation was carried out for different times (0, 10, 20, 40, 60, 80 min) using a Phillips UV lamp (9 W/cm²), mounted at 8 cm distance from the surface. Polymer grafting was also performed with the addition of TED at the concentrations 0, 100 nM, 1 μ M, 10 μ M and 100 μ M. Experiments were repeated in a modified atmosphere, inside an Atmosbag continuously purged with nitrogen. The grafted supports were washed with methanol, dried and analysed with contact angle (CA), AFM and FT-IR.

2.8. Fluorescent analysis of the grafted layer

The fluorescent monomer 9-anthracenylmethyl acrylate in the concentrations 0%, 0.031%, 0.0625%, 0.125%, 0.25%, 0.5% and 1% was added to a solution containing 0.1 g MAA, 0.9 g EGDMA, in 1 g toluene, sonicated for 10 min and then purged with argon for 20 min. The slides with the initiator attached to the surface were placed in covered Petri-dishes and immersed in the polymerisation mixtures. The covalent attachment occurred under UV light during 20 min. The surfaces were then washed with methanol and dried under a stream of nitrogen.

Quantification of the fluorescence of the surface was performed with a fluorescence analyser equipped with GeneSnap and GeneTool softwares (Syngene Europe, Cambridge, UK). Images were captured at fixed readout wavelength (λ_{exc} 320 nm, λ_{em} 410 nm), 0.200 s exposure, 20 RU radius. A calibration curve was plotted with the relative fluorescence units (RFU) and the quantity of monomer added to the mixtures. The equation for the calibration was linear, $y(RFU) = 57865x(\mu\text{g of fluorescent monomer})$. The fluorescence readouts were converted into $\mu\text{g/area}$ and into mol/area.

2.9. FT-IR analysis of the grafting and AFM analysis

FT-IR spectroscopy was used for the qualitative analysis of the grafted polymers. The equipment used was a Bruker IR-Scope-II (Bruker Optics, UK) equipped with attenuated total reflection (ATR) objective and controlled by Opus IR optic software. FT-IR spectra were collected in reflectance mode, in the range between 600 and 4000 cm⁻¹, subtracted for the background. The different preparation steps were analysed. AFM images were obtained in contact mode using the PicScan™ instrument (Molecular Imaging Corporation, Tempe, AZ, USA). Surface topography was analysed by scanning regions of 10 $\times$ 10 μm^2 and 1 $\times$ 1 μm^2 .

2.10. Ellipsometry measurements

A Reaglehole Instruments phase-modulated Picometer ellipsometer (He-Ne laser, $\lambda = 632.8$ nm) was used to measure the thickness of dry layers of the grafted polymers.

A refractive index of 1.48 was used for polymer layers. The ellipsometric angles Ψ and Δ were measured at angles of incidence ranging from 80° to 35° (measurements were made every 1.00°), and fit using a Cauchy model (Igor Pro. software package) to obtain the thickness with an accuracy of ± 1 nm. Thickness measurements were taken at five different points on every sample measured in ambient air.

3. Results and discussion

3.1. Derivatisation of the supports with iniferter

The silane iniferter *N,N*-diethylaminodithiocarbamoylpropyl-(trimethoxy)silane was synthesised with a modified protocol adapted from Kobayashi et al. (1992) (Appendix B Supplementary

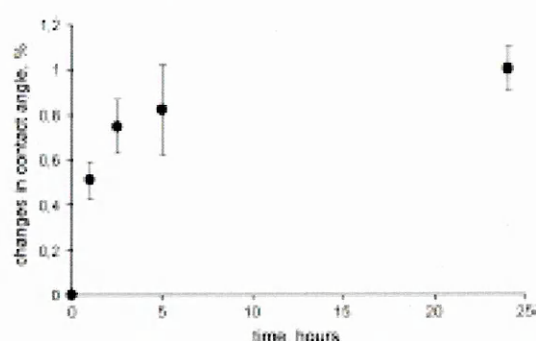


Fig. 1. Time course of the derivatisation of borosilicate supports with silane living iniferter.

material 1). Advantages of the modified protocol are the ease of purification and high yield (76%) of product.

Support surfaces were modified with the silane living iniferter after activation. The time course of the modification was studied and optimised by static CA measurements. Bare silica has a CA of 10–12° that changed to ca. 80° due to the modification. The changes in CA measured were plotted as percentage versus time, as reported in Fig. 1. Results displayed a saturation course and were in line with the literature (Kobayashi et al., 1992). A period of 3 h was considered satisfactory for the derivatisation process.

An estimation of the quantity of iniferter groups placed on the supporting surface was attempted by using fluorescence, in line with other quantitative fluorescence surface studies (Ivanov et al., 1996; Xing and Burguet, 2007). It was supposed that the activation of the iniferter groups in the presence of fluorescent monomers would result in labelling the majority of the iniferters, thus offering the possibility to estimate the quantity of active iniferters on the surface. The photo-activation was allowed to proceed for different times (20 and 40 min) on supports derivatised with silane iniferter and on controls. The results are summarised in Table 1 with batches showing variability in the range 10–15%, possibly due to self-aggregation of the fluorescent monomer as a source of error. Data for 20 or 40 min photo-activation did not vary significantly, probably indicating that the grafting of the layer of monomers onto the surface is a fast process that occurs within the first few minutes. Longer irradiation times resulted greater variability in the results, presumably due to other phenomena, such as self-polymerisation of the monomers in solution, or loss of iniferter groups at the surface occurring, thus confusing the experimental picture. Upon ultrasonication the quantity of active iniferter groups attached to the surface dropped by 50% with respect to non-ultrasonicated supports, suggesting that loosely attached monomers and iniferter are removed in the process. The quantity of grafted iniferter groups was estimated as 20–30 pmol/mm².

3.2. Polymer grafting

A layer of polymer was grown from the surface. The activation of the polymerisation process was by UV photodecomposition of the iniferter attached to the support. Ellipsometry measurements indicated that 20 min UV irradiation gave rise to ~5 nm polymer thickness, 40 min UV gave rise to ~6 nm polymer thickness. Time seemed scarcely relevant for determining the thickness of the grafted layer and no variations were detected between the different monomer compositions tested. CA measurements were reported in Fig. 2A, showing an angle of about 68° for the grafted polymer, which is in accordance with the literature data (Yuan et al., 2007).

Table 1
Quantity of active iniferter on the surface.

Sample description	UV exposure (min)	Ultrasonication after polymerisation	Fluorescent monomer ($\mu\text{g}/\text{mm}^2$)	Fluorescent monomer ($\mu\text{mol}/\text{mm}^2$)
Control	20	No	0.012 ± 0.002	40 ± 7
Iniferter	20	No	0.021 ± 0.003	74 ± 11
Control	40	No	0.011 ± 0.001	45 ± 4
Iniferter	40	No	0.033 ± 0.002	87 ± 5
Control	20	5 min	0.005 ± 0.001	19 ± 4
Iniferter	20	5 min	0.007 ± 0.001	28 ± 4

In order to characterise the grafted layers, FT-IR spectra of the supports after each derivatisation step were collected. Fig. 2B shows the spectra of the iniferter-modified support and of the grafted polymers P3, P4 and P5. Iniferter-modified supports displayed a characteristic peak at 1650 cm^{-1} for the C=S group and some peaks in the region around 1200 cm^{-1} , attributed to thiocarbonyl (C=S) stretching (Fig. 2B (a)). As expected, the contribution of the dithiocarbamate iniferter, including the presence of CH_3 and CH_2 vibration is seen between 1100 and 1550 cm^{-1} (Han and Williams, 1991).

In the case of polymers P3, P4 and P5, the wavenumber of the C=O stretching vibrations in the region of 1900 – 1200 cm^{-1} is a function of the copolymer composition (Ruckert et al., 2002). The spectra showed the C=O stretching vibration, typical of the ester/acid carbonyl groups, at $\sim 1732/1710\text{ cm}^{-1}$ (Fig. 2B, arrows (b)). The asymmetric carboxylate stretching band $\nu_{\text{as}}(\text{COO}^-)$ is observed near 1560 cm^{-1} (Fig. 2B (c)) (Colthup et al., 1975).

Polymer P3 contained methacrylic acid with CH_3 and CH_2 groups giving stretching absorptions at 2998 and 2954 cm^{-1} , respectively. The deformation bands of these groups were observed at 1487 and 1452 cm^{-1} . The C=O stretching around 1700 cm^{-1}

was also observed. Polymer P4 contained the trifluoromethyl acrylic monomer distinguished by the presence of intense signals at 1250 cm^{-1} (Fig. 2B (d)). Polymer P5 contained the phosphate monomer, which gave an intense signal in the region 1250 – 1350 cm^{-1} (Fig. 2B (f)). These results prove the different natures of the grafted polymers.

3.3. Growing grafted polymers with controlled thickness

The goal of this work was to achieve control over the layer of cross-linked polymer grafted to the surface. Producing a grafted polymer of a defined thickness has practical importance, e.g. in assuring the formation of an adequate number of binding sites, as in the case of polymer used in sensor development.

There are different factors responsible for polymer growth and therefore important for controlling the process and extending the grafted layer of significant dimensions: (i) the amount of iniferter on the surface (Lin, 2001), (ii) the reactivity of the monomer and the cross-linker (Ward et al., 2002), (iii) the stability of the radicals (Ward et al., 2002), (iv) the decrease of the polymerisation rate at the surface due to diffusional and steric barriers (Blaya et al., 2004), (v) the presence and the incorporation of oxygen in the solution during the polymerisation, (vi) the equilibrium between active/dormant species (Mijangos et al., 2005).

We took into consideration the presence of oxygen and the equilibrium between active/dormant radical species. Fig. 4 indicated that an oxygen-free environment is essential for the growth of the polymer. The thickness of P3 obtained from solutions simply purged with argon was 6 nm , while polymerisation in better-controlled atmosphere within an Atmosbag produced a coating of 20 nm thickness.

In the second case, the equilibrium between active/dormant radicals was shifted by the addition of a chain terminating agent, TED (Otsu, 2000). The terminating agent TED was added to the monomer mixture at the concentration of $10\text{ }\mu\text{M}$ and polymerisation was carried out for 80 min . Ellipsometry was used to determine the thickness of the grafted polymer layer. Results shown in Fig. 3A indicated that P3 reached the significant thickness of ca. 100 nm . It could be hypothesised that $10\text{ }\mu\text{M}$ of TED in the monomer solution would prevent the irreversible recombination of active radicals by providing an excess of dormant radicals, thus prolonging the lifetime of the surface-grafted macroiniferter.

The influence of the polymerisation time on polymer thickness was evaluated. Fig. 3B shows that increased irradiation time corresponds with an increased thickness of the grafted polymer layer. It was noted that 40 min polymerisation produced a thickness of about 70 nm , while 80 min produced a 100 nm layer. The relationship between irradiation time and thickness of the grafted layer was almost linear (equation: $y(\text{nm}) = 1.3857x(\text{min of polymerisation})$; $R^2 = 0.8952$), this observation is in accordance with other surface macromolecular architectures prepared by photochemical initiation (Nakayama and Matsuda, 1996) and accounts for a limited disruption of the surface proximity to the chain elongation process. Further investigations were conducted to determine whether

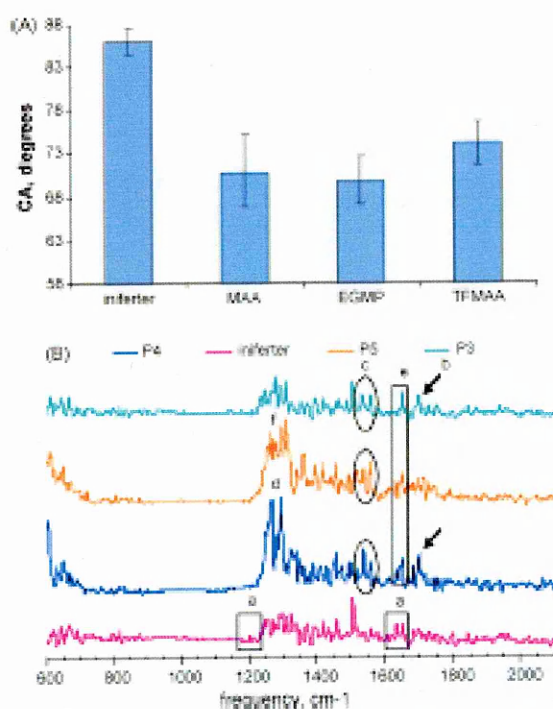


Fig. 2. (A) Contact angle measurements as evidence for the grafting of the different polymeric compositions. (B) FT-IR spectra of the different polymers.

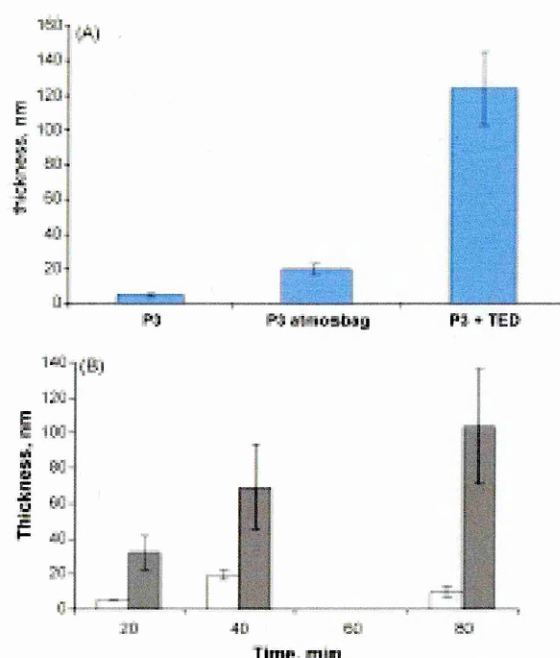


Fig. 3. (A) Thickness of the grafted polymer layer. (B) Time dependence of the thickness of the grafted layer. Solid bars indicate P3 + TED 10 μM. White bars indicate control experiment in the absence of TED.

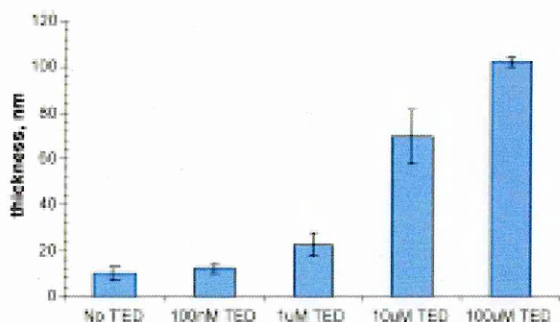


Fig. 4. Relationship between the concentration of TED in solution and the thickness of the grafted layer.

there was a correlation between the concentration of TED and the thickness of the grafted polymer. Fig. 4 shows the dependency of the grafted polymer thickness on the concentration of the chain terminating agent, TED. The irradiation time was fixed at 40 min. A direct correlation between the concentration of TED and thickness of polymer emerged, indicating that manipulation of the quantity of TED in solution is an effective way to obtain grafted layers with thicknesses between 5 and 130 nm. A plateau effect seemed to occur for high concentrations of TED. The data were supported by FT-IR and AFM studies.

4. Conclusions

In conclusion, the versatility of living initiators to prepare grafted layers was recently demonstrated in the work on functionalised nanoparticles, including molecularly imprinted polymers (Ruckert et al., 2002). Subsequently, the possibility to polymerise consecutively different layers, each one incorporating different

properties as desired was also demonstrated (Pérez-Moral and Mayes, 2007). Control of polymerisation allows for the design of customised multiple-functionality layers, one on the top of the other and offers practical solutions for biosensors and biomimetic applications on chips.

In order to understand and have control of the growth of cross-linked polymeric surfaces, our research covered the basic aspects of the production of polymer surfaces using living iniferter mediated polymerisation. Monitoring each preparation step and the polymerisation with FT-IR allowed a close control of the grafting process as the distinctive vibrational signals for iniferter and for polymeric materials were identified. The grafting was directly dependent on the irradiation time and on the concentration of chain terminating agent. The best conditions for grafting so far identified were 40–60 min of irradiation and 10–100 μM of TED, which allowed the growth of a 100 nm thick polymer layer, while a 100 nM TED concentration resulted in the formation of 10 nm thick polymer film. The process could in principle be extended to any composition of polymer desired, including MIPs. Such an approach would help to design ideal or highly compatible recognition layers to be integrated within sensor systems, optimising electrical conductivity and reducing diffusion times for the analytes, thus enabling the preparation of a new generation of highly sensitive polymer-based sensors. In conclusion we have demonstrated an ability to enhance control over the thicknesses of polymeric coating made of cross-linked materials.

Acknowledgements

Authors thank EU-Marie Curie TOK-IMP NanoDAT project and Fondazione Cariverona.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.sbe.2010.02.015.

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HIGHLY SENSITIVE CANCER BIOMARKER DETECTION IN HUMAN SERUM (75%) USING REAL-TIME LABEL-FREE BIOSENSORS

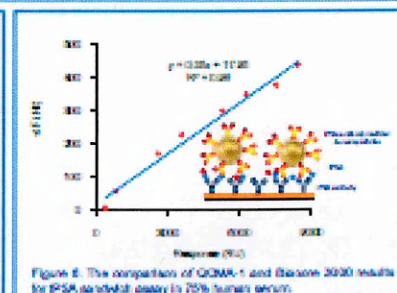
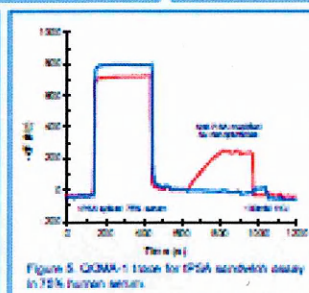
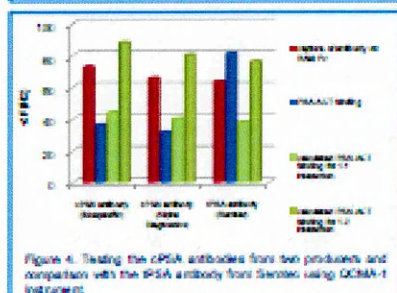
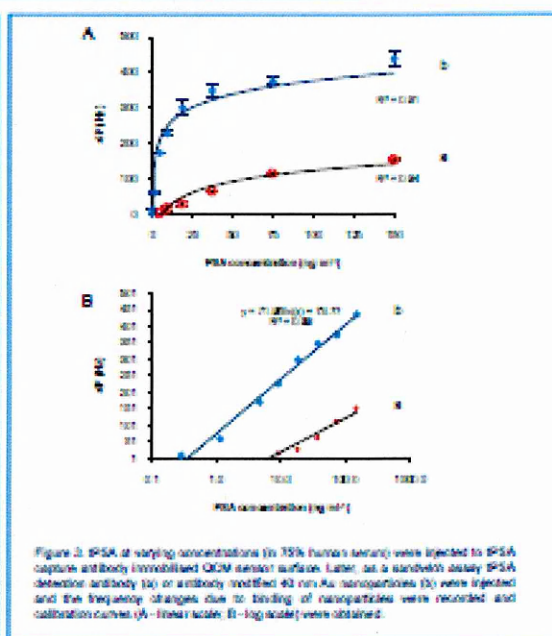
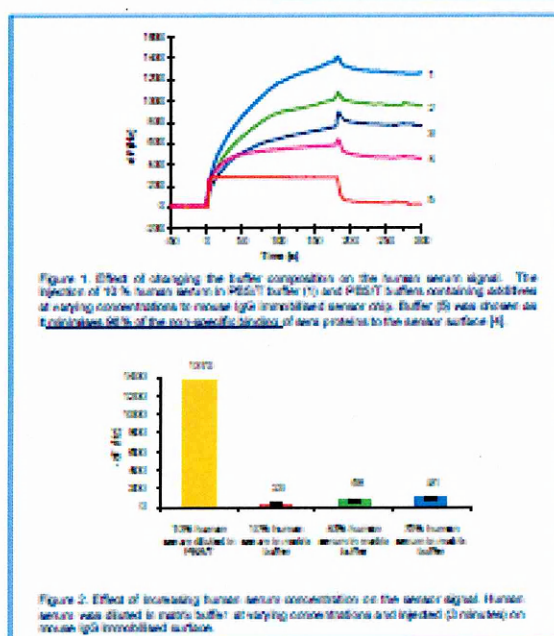
Yildiz Uludag, Ibtisam E. Tothill

Advanced Diagnostics and Sensors, Cranfield Health, Cranfield University, Cranfield, Bedfordshire, MK43 0AL, UK

Introduction

There is a significant difference in the limit of detection when buffer or clinical sample is used as the assay media for biosensor assays. The change in the detection limit, when clinical samples are used, is mainly due to the lower signal to noise ratio resulting from the high non-specific binding of biomolecules (found in high concentrations in clinical sample) to the sensor surface. This is especially noticeable when label-free biosensors are employed. Therefore researchers avoid the use of high concentrations of serum in most analysis and serum concentrations of 10 to 50 % were usually reported [1-3]. Two label-free biosensor platforms, a novel, fully automated QCM biosensor (QCM-A-1, Sierra Sensors GmbH, Germany) and an SPR biosensor (Biacore 3000, GE Healthcare, Sweden) for biomarker detection in human serum were used in this study. Prostate specific antigen (PSA) was chosen as the cancer biomarker to be detected in serum.

Methodology and Results



Conclusion

Initially a new buffer was formulated to eliminate 98% of the non-specific human serum protein binding to the sensor surface. Using this new formulated 'matrix buffer', the detection of PSA concentrations down to 0.29 ng ml^{-1} in 75% serum was achieved with a linear detection range of $0.29 - 150 \text{ ng ml}^{-1}$ using the QCM and the SPR sensor chips with Au nanoparticles employed for sensitivity enhancement [4, 5]. The achieved results indicate that data obtained from both QCM-A-1 and Biacore 3000 instruments were comparable and additionally both sensor chips can be used for the fast and easy detection of PSA in patient samples.

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Author contribution: We gratefully acknowledge Sierra Sensors GmbH for providing financial support for the study.

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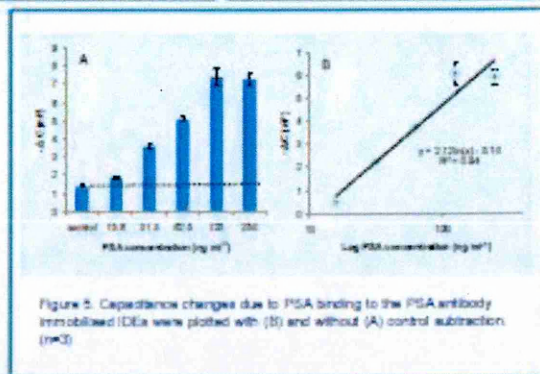
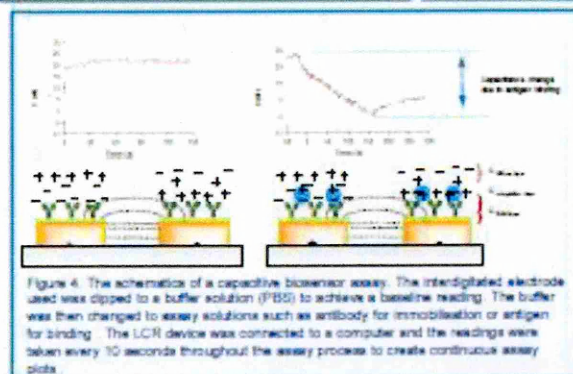
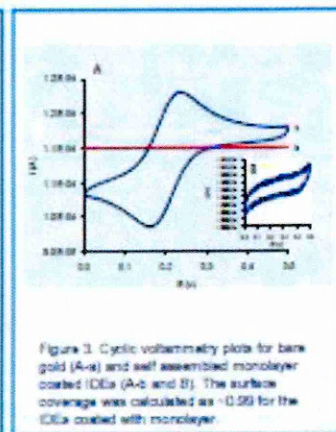
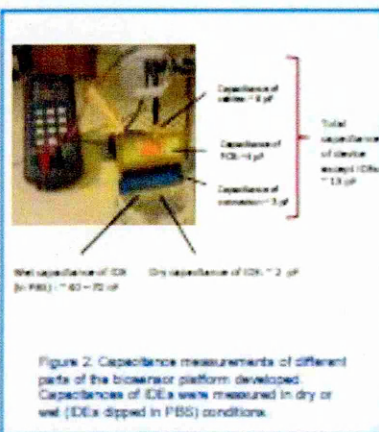
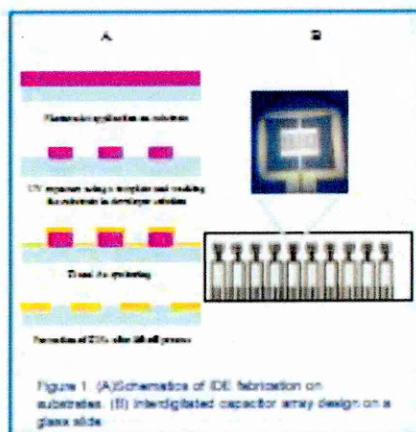
Development of a novel capacitance biosensor for biomarker detection

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Introduction

In this investigation, interdigitated electrode (IDE) based capacitive, label-free biosensor arrays were designed, fabricated and used for the analysis of prostate cancer biomarker. The detection and quantification principle of the capacitance biosensor, in this study, was based on the measurement of changes in dielectric properties and charge distribution due to affinity interactions taking place on the surface of the electrode array. Two arrays of electrodes with comb shape were designed and utilised in this work. Nine gold-IDEs were fabricated using conventional micro-fabrication technologies on each standard glass slide. An antibody against prostate specific antigen (PSA) as a cancer biomarker was immobilised onto the IDE surface. Antigen binding assays were then developed to capture the PSA molecule achieving a detection limit of 15.6 ng mL^{-1} with a linear dynamic detection range of $15.6 - 250 \text{ ng mL}^{-1}$ PSA when a real-time, continuous assay was employed. This study clearly demonstrates the potential use of the label-free and real-time capacitance-sensor platform for point-of-care testing. Further developments are underway to optimise the system using the necessary electronic detection units.

Methodology and Results



Discussion & Conclusion

A novel IDE array and PCB were designed and fabricated and the capacitance reading was performed using a simple LCR meter. This new prototype enabled both consistent capacitance reading and also enabled the real-time detection to be performed in buffer. Antigen binding assays were developed to capture the PSA molecule in buffer solutions achieving a detection limit of 15.6 ng mL^{-1} with a linear dynamic detection range of $15.6 - 250 \text{ ng mL}^{-1}$ PSA. Now a new prototype is in the process of being designed and fabricated that enables multiplexed, real-time, continuous measurement of analytes. Additionally further developments are underway to optimise the system using the necessary electronic detection units, in order to enhance the sensitivity of the system.

References

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Acknowledgements

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POINT-OF-CARE TESTING FOR PROSTATE CANCER DIAGNOSIS

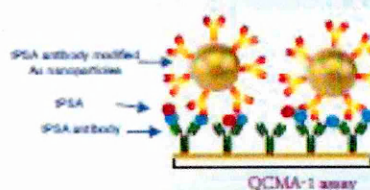
Y. Uludag, IE. Tothill*

Cranfield Health, Cranfield University, Cranfield, Bedfordshire, MK43 0AL, UK

Introduction

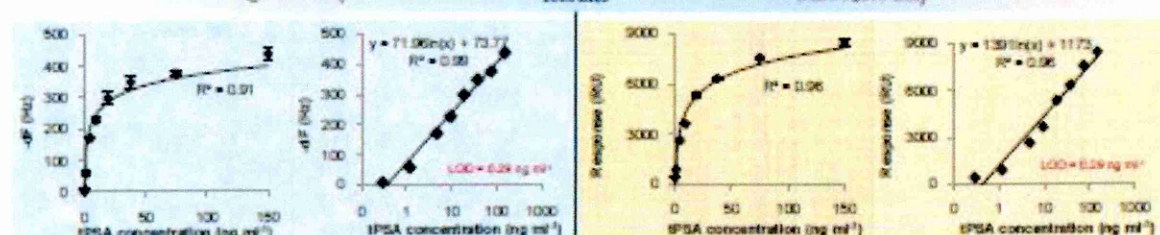
Prostate carcinoma is a fatal malignancy and is a major cause of death in men in the population aged 55 and over. Prostate cancer is the commonest form of cancer in men in Europe with 301,500 incident cases a year that corresponds to 24.1% of all cancer cases¹. Early and accurate detection of prostate cancer is very important, since there is no cure if the cancer spreads out to the other organs of the body. The increase in prostate specific antigen (PSA) levels in serum above the normal limits (4 ng ml^{-1}) is the primary indication of prostate malignancy; therefore PSA is used as a biomarker for the diagnosis and prognosis of the prostate cancer^{2,3}. ELISA type PSA tests are usually performed at centralised laboratories using expensive automated analyzers and time consuming procedures⁴. This process requires sample transportation, increased waiting time and high medical costs. Therefore there is a need to develop a detection system that is cost effective, quick to process, uses low sample and reagent volume and easily operated. Biosensor technology has the potential to use validated biomarkers for the development of point-of-care devices⁵. In this study the suitability of a QCM and an SPR biosensor were investigated for tPSA test.

Methodology

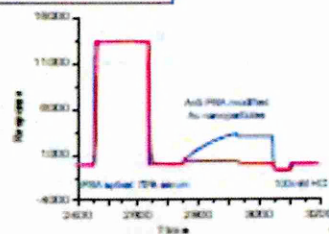
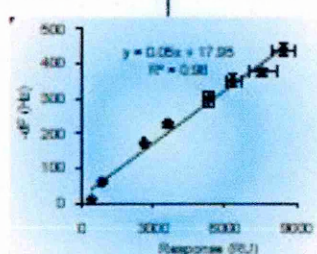
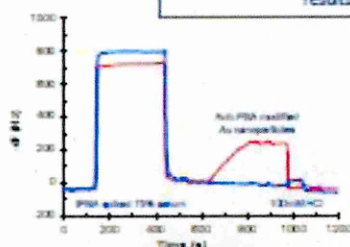


A QCM biosensor (QCM-1; Sierra Sensors GmbH, www.sierra-sensors.com) and an SPR biosensor (Biacore 3000, GE Healthcare) were used for tPSA (total PSA) detection. Initially a matrix elimination buffer containing additives was formulated. This buffer was designed to reduce the non-specific binding of sera proteins to the sensor surface by 98%. Concentrations of tPSA were prepared as 50% PSA-ACT(complexed PSA) and 50% PSA (free PSA). A sandwich assay was performed using tPSA detection antibody modified Au nanoparticles for signal amplification. After the bindings 100 mM HCl ($\approx 20 \text{ mM}$ NaOH for high concentrations of PSA) were used to regenerate the sensors.

Results



Calibration curves for the detection of tPSA that is spiked in 75% human serum. Sandwich assay results using tPSA detection antibody modified Au nanoparticles.



The comparison of QCM-1 and Biacore 3000 results for tPSA sandwich assay in 75% human serum.

Discussion & Conclusion

Free PSA (direct) binding to the anti-PSA capture antibody data fitted to 1:1 Langmuir binding model and K_D calculated as $9.46 \times 10^{-12} \text{ M}$ using Biacore 3000 and $5.56 \times 10^{-12} \text{ M}$ using QCM-1 instruments⁶. tPSA detection assay in human serum resulted in detection of tPSA concentrations down to 0.29 ng ml^{-1} in 75% serum with a linear detection range of $0.29 - 150 \text{ ng ml}^{-1}$ using both instruments when 40 nm Au nanoparticles were employed to enhance the sensitivity. The tPSA concentration of male human serum (from Sigma) used in this study was calculated as 0.41 ng ml^{-1} using QCM-1 and 0.56 ng ml^{-1} using Biacore 3000 instruments. These results indicate that data obtained from both QCM-1 and Biacore 3000 instruments are compatible with each other and additionally both instruments can be used for the fast and easy detection of tPSA in patient samples.

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POINT-OF-CARE TESTING FOR PROSTATE CANCER DIAGNOSIS

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UNIVERSITY

Introduction

Prostate cancer (Pca) is a common disease in elderly men. In the UK, about 10,000 men die of it annually. Early and accurate detection of biomarkers of Pca development and progression will help prevent or delay the onset of incurable metastatic Pca. Biomarkers such as androgen and oestrogen receptors, MGMTs, TMPRSS fusion transcripts with ERG and / or ETV1, E-Cadherin 1, FASN, CD44, Aurora kinases (A,B and C), BCL-2, KLK2, BCRA2, TP53, and KLK3 were evaluated using real time PCR. The markers are considered for point-of-care testing (POCT). Recently, biomarkers such as sarcosine, uric acid, kynurenine, glycerol-3-phosphate, leucine and proline were reported to have strong association with Pca progression. However, serum PSA testing, digital rectal examination and histological analysis of sextant prostate biopsies are the core clinical diagnostic tools despite their shortcomings. In addition, PCA3 (DD3) is now CE-marked for clinical laboratory screening of prostate cancer.

Advances in sensor technology have enabled the miniaturisation of devices and multiplex testing of analytes in urine, blood and/or tissues. Biosensor technology has the potential to use validated biomarkers for the development of POCT device. Rapid sensors based on multi-analytes' chips are being applied in this work to develop a POCT device for prostate cancer diagnosis.

Methodology

Detection of biomarkers using real time PCR

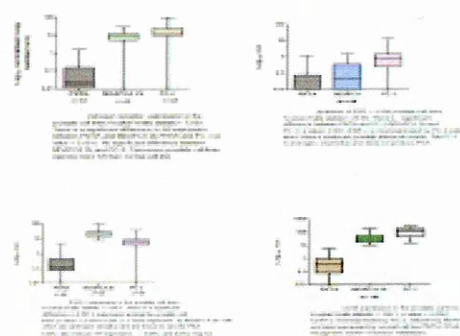


Development of a biosensor for prostate cancer detection

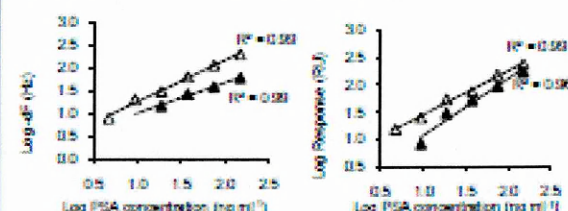
A quartz crystal microbalance biosensor (QCM-A1) and a surface plasmon resonance biosensor (Biacore 3000) were used for the optimisation of PSA detection. PSA was used as the model marker compound to develop the affinity biosensor. Initially a new buffer with additives was designed to lower the non-specific binding of sera proteins to the sensor surface, then the assay performed in buffer and in human serum spiked samples.



Results



The markers were first tested in hormonally modulated Pca cell lines and there was differential expression of the normalized ratios. Some of the markers were also detectable in urine sediments of healthy subjects.



Calibration curves for the detection of PSA that is spiked in 75% human serum and in buffer. Sandwich assay results using anti-PSA detection antibody in buffer (triangle) and in spiked serum (solid triangle).

PSA binding to the anti-PSA capture antibody data fitted to 1:1 Langmuir binding model and KD calculated as 8.45×10^{-11} M using Biacore 3000 and 5.56×10^{-11} M using QCM-A1 instruments. PSA detection assay in human serum resulted in detection of PSA concentrations of 18.8 ng ml^{-1} using both instruments.

Discussion & Conclusion

Of the 15 molecular markers evaluated in early morning urine sediments of 30 healthy subjects, TMPRSS fusion transcripts (ERG and ETV1) were undetectable. The other markers: MGMT, MGMTs, ERG and ETV1 receptors were very low: 5 to 35 copies. AR was highly expressed in healthy subjects. KLK2 and KLK3 were also detectable. Bcl-2, TP53, E-cadherin and CD44 were very low (<20 copies). The gene expression was normalized with ABL1 reference gene. Real time PCR on urine sediments of BPH and Pca patients are still being conducted. A multiplex qPCR is designed to test in a single tube five of the most differentially expressed markers and a control gene for further validation. The targets will be considered for a multi-analyte biosensor development.

PSA assay has been chosen as a model system to optimise the biosensor assays. Both, the SPR based sensor, Biacore 3000 and the QCM sensor, the QCM-A1, showed similar sensitivity to the PSA assay (18.8 ng ml^{-1} in 75% serum). The current detection limit has been improved recently (data not shown) through the application of nanoparticles to the assay system. The current system will be developed for non-invasive Pca diagnosis.

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Prostate Cancer Diagnosis Using an Affinity Sensor

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ABSTRACT

Prostate carcinoma is a fatal malignancy and is a major cause of death in men in the population aged 55 and over in the UK (10,000 men a year). Early and accurate detection of prostate cancer is very important, since there is no cure if the cancer spreads out to the other organs of the body. Recent developments and advances in biosensor technology have enabled the miniaturisation of the devices and multiplex testing of the analytes. Therefore, biosensor technology has the potential for the manufacture of point of care cancer testing devices.

Prostate specific antigen (PSA) is used as a biomarker for the diagnosis and prognosis of prostate cancer. Tests based on PSA analysis are currently still the most common tests employed for prostate cancer detection. In the current study the potential of using QCM biosensors for the detection of PSA has been investigated. An automated QCM instrument was used as the biosensor platform. By performing a direct immunoassay on the gold sensor chip a 19.5 ng ml⁻¹ PSA could be detected, while a sandwich assay enabled the detection of 4.9 ng ml⁻¹ PSA.

INTRODUCTION

Prostate cancer, the commonest form of cancer in men in Europe, is a complex, multifactorial disease. Every year in the UK nearly 85,000 cases of prostate cancer are diagnosed, and prostate cancer causes 12,000 deaths each year.

(<http://info.cancerresearchuk.org/cancerstats/types/prostate/incidence/>). Early and accurate detection of prostate cancer is very important, since there is no cure if the cancer spreads out to the other organs of the body. Early diagnosis of prostate cancer is very important for successful treatment of the disease.

The increase in PSA levels in serum above the normal limits is the primary indication of prostate malignancy; therefore PSA is used as a biomarker for the diagnosis and prognosis of the prostate cancer (Brawer and Lange 1989; Diamandis 1998). PSA or in other words human glandular kallikrein 3 (hK3) is a 33-34 kDa glycoprotein produced by the prostatic secretory epithelium cells. PSA is also detectable in serum and the normal level of PSA in serum is 0.1-2.5 ng ml⁻¹. In serum, PSA is found either in free form or as a complex with ACT (ACT-PSA, MW 96 kDa). ACT is a protease inhibitor and it inhibits PSA's proteolytic activity. Although PSA is currently recognised as the clinically most used prostate cancer marker, it is not cancer specific (Stephan, et al. 2007). Benign Prostatic Hyperplasia

APPENDIX B - ETHICAL APPROVAL FORMS

Patient Information Sheet

1. Study Title

Hormonal Modulation of Putative Tumour Markers in Prostate Cancer.

Microarray sensor for prostate cancer diagnosis.

2. Invitation Paragraph

You are being invited to take part in two research studies. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss with friends, relatives and your GP if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. Consumers for Ethics in Research (CERES) publish a leaflet entitled 'Medical Research and you'. This leaflet gives more information about medical research and answers some questions you may want to ask. A copy may be obtained from **CERES, PO Box 1365, London N16 0BW**.

3. What is the purpose of the study?

The natural course of most prostate cancers is unpredictable. Some patients who have indolent prostate cancers progress to symptomatic forms, while others don't. Some progress further to metastatic cancer, which are more aggressive and lethal. There are little or no factors to discriminate between 'good' and 'bad' prostate cancer. This study examines the expression of several types of oestrogen receptors, which are crucial to prostate cancer progression and may also indicate potential aggressive forms and inform choice of therapy. The receptors are investigated alongside with other generally recognised markers of prostate cancer.

In the second study a microarray sensor will be developed to diagnose prostate cancer. The current prostate cancer diagnosis method involves highly invasive treatments like digital rectum examination and biopsy. The aim is to develop a new biosensor that will detect the biomarkers of the prostate cancer from blood or urine samples. This new biosensor is expected to predict the condition of the patient better than the current methods so that the number of patients that need to send to the biopsy will decrease.

4. Why have I been chosen?

As you are aware, your clinician has informed you that a biopsy is going to be taken from your prostate as you have been shown to have a raised PSA and / or an enlarged prostate. If you consent to taking part, a urine sample and a blood sample will be collected before your biopsy. Once your biopsy has been examined, any remaining tissue will be allocated to the study.

5. Do I have to take part?

It is up to you to decide whether or not you wish to take part. If you decide to take part, you are still free to withdraw at any time without any reason. This will not affect the standard of care you receive.

6. What happens to me if I take part?

If you decide to take part, you will be asked to sign a consent form for this study. Before your biopsy is taken, you will be asked to give a urine sample and a blood sample. When the biopsy has been looked at for your diagnosis, part of the remaining tissue will be allocated for this study.

7. What do I have to do?

We ask you to carefully read this information and take your time in deciding if you want to be part of this study. If you agree to take part, you'll be asked to sign a consent form and give a urine sample. When the routine biopsy is

taken the normal procedure is performed and part of any remaining tissue is allocated for this study.

8. What is the drug or procedure that is being tested?

No drug or procedure is being tested.

9. What are side effects of taking part?

There are no risks associated with collection of urine sample. The biopsy has no additional side effects apart from those normally associated with the operation. Taking a blood sample may cause slight discomfort and sometimes you can get localised bruising around the area from which the sample was obtained.

10. What are the possible disadvantages and risks of taking part?

There are no possible disadvantages or risks associated with taking part in this study.

11. What are the possible benefits of taking part?

There is no direct benefit to you but this study may help improve diagnosis and future treatment of prostate cancer.

12. What happens when the research study stops?

All tissue allocated for this study will be destroyed and the results will form the bases of a scientific thesis and may generate scientific publications.

13. What if something goes wrong?

If you are harmed by taking part in this research, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain about any aspects of the way you

have been approached or treated during the course of this study, the normal National Health Service complaints mechanism may be available to you.

14. Will my taking part in this study be kept confidential?

All information that is collected about you during the course of the research will be kept strictly confidential. Any information about you which leaves the hospital/surgery will have your name and address removed so that you cannot be recognised. All samples will be analysed anonymously.

15. Who is organising and funding the research?

The research is being organised and funded by Cranfield University.

16. Who have reviewed the study?

Cranfield health local ethics committee, Bedford Hospital NHS trust research and development unit and Bedford Hospital NHS trust medical research ethics committee

17. Contact for further information

Dr. M Wasil

Department of Research and Development

Bedford Hospital

Kempston Road,

Bedford, MK42 9DJ.

Tel: 01234 355122 Ext. 5855

Mohammad.Wasil@bedfordhospital.nhs.uk

STUDY PROTOCOL: Microarray sensor for prostate cancer diagnosis.

Different prostate cancer biomarkers will be identified to be used for the microarray biosensors. Initially the microarray sensor will be optimised using biomarkers in buffer solutions and in serum.

Sample Collection: Blood samples will be collected by the medical professional (10 mL from each volunteer). Blood sample will be transferred and stored at -20°C in the Molecular Biology lab of Cranfield Health.

Analysis: Blood samples will be analysed using the microarray biosensor developed to identify the concentration of prostate specific biomarkers.

Results: Results from laboratory assays will be statistically analysed and used in writing up PhD thesis and may also be published in peer-reviewed journal.

Anonymity: No patient-identifiable information is required at any stage of this work including sample collection. The nature of sample collection for this study does not require informed consent from patients. No genetic testing will be done. The log book for sample collection and results of laboratory analysis will be stored in a password protected computer.

Centre: Bedford Hospital NHS Trust
REC Reference Number: 08/H0311/90
Patient identification Number for this study:.....

CONSENT FORM

Title of Project: *Hormonal modulation of putative markers in prostate cancer and microarray based sensor for prostate cancer diagnosis.*

Name of Researchers: *Emmanuel Nna & Yildiz Uludag.*

(Please initial box)

- 1. I confirm that I have read and understood the information sheet dated 12/06/08, version 01/08 for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.
 - 2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected.
 - 3. I understand that relevant sections of any of my medical notes and data collected during the study may be looked at by responsible individuals from Cranfield University, from regulatory authorities or from NHS trust, where it is relevant to my taking part in this research. I give ermission for these individuals to have access to my records.
- 1. I agree to my GP being informed of my participation in the study.
 - 2. I agree to take part in the above study.

.....
Name of patient Date Signature

.....
Researcher Date Signature

Letter of invitation

Dear Participant,

We invite you to participate in a study on prostate disease. This study looks at some indicators of prostate disease development and progression. Our aim for the study is to examine the link between the development of prostate disease and the production of these indicators, also known as disease markers. Results from this study may help improve diagnosis, treatment and management of prostate disease in the future. As you are aware, your clinician has informed you that a biopsy is going to be taken from your prostate as you have been shown to have a raised PSA and / or an enlarged prostate. The biopsy is part of your clinical care and will be used by the hospital laboratory to find out if you have prostate problem. The left over tissue will be used for this study. If you consent to taking part, urine and blood samples will be collected from you before your biopsy.

More details of this study and your participation are contained in an information sheet also given to you. Please take time to read the information carefully and discuss with friends, relatives and your GP if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. There is a letter for your GP, informing him or her about your participation in this study. Please kindly give the letter to you GP. Summary of results from this study will be made available to you and your GP. You are also given a consent form. If you decide to take part in this study, you will be asked to sign a consent form for this study. Before the Urologist takes your biopsy, you will be asked to give a urine sample and a blood sample, which will be collected by a nurse. We thank you for taking time to read this.

Yours Sincerely,

Emmanuel Nna and Yildiz Uludag

Letter to General Practitioner

Cranfield Health
Building 52,
Cranfield University
Bedfordshire,
MK43 0AL.
15-08-08.

Dear GP,

Hertfordshire REC Reference Number: 08/H0311/90

Study Title: Indicators of Prostate Disease

Your patient, MR..... is invited to participate in a study on 'indicators of prostate disease'. The study examines disease markers that can be used to identify 'bad' prostate disease. As you are aware, your patient has raised serum PSA level and or enlarged prostate gland and has been scheduled to undergo prostate biopsy. If he consents to this study, blood and urine samples will be collected from him before clinical biopsy.

Find enclosed a copy of patient information sheet, patient invitation letter and consent form, which are also given to your patient.

Results of this study will be made available to you and your patient through the collaborating clinician at the Bedford hospital (Dr.H Sharma). The results of this research will also be used in writing a PhD thesis, which will be published on line in the Cranfield University website: www.cranfield.ac.uk

If you have any concern, please do not hesitate to contact me on phone: 01234 75 5561 or e-mail:

e.o.nna.s05@cranfield.ac.uk .

Yours Sincerely,

Emmanuel Nna and Yildiz Uludag