CRANFIELD UNIVERSITY

MARIA PAWULA

DEVELOPMENT OF A SENSITIVE IMMUNOSENSOR FOR THE DETECTION OF CARDIAC TROPONIN T IN CARDIOVASCULAR DISEASE

CRANFIELD HEALTH

MSc Thesis

For MSc by Research
Academic Year: 2013 - 2014

Supervisors: Prof. Ibitsam E. Tothill
Dr Zeynep Altintas
Dec 2014

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Advanced Diagnostics and Sensors Group

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ABSTRACT

Cardiovascular disease (CVD) is currently globally the biggest cause of mortality, with rising figures, especially now in the developing world. Early and accurate diagnosis of CVD, (especially acute myocardial infarction (AMI) is important in being able to provide appropriate, timely and cost effective treatment, or to take preventative action. Biomarkers and biosensors are playing an increasingly important role in this diagnosis, especially those based on immunoassays. As technology improves and becomes cheaper, there is the potential to develop immunosensors which use optical techniques such as surface plasmon resonance (SPR) for biomarker measurement which could be used effectively in point-of-care diagnostics for real-time detection.

This thesis describes the development and optimisation of a sensitive immunosensor for the AMI specific biomarker, cardiac Troponin T (cTnT), on an SPR platform. Early diagnosis of AMI requires an assay methodology which can determine very low concentrations of cTnT in human serum.

The work conducted includes the development of a set of optimised conditions for the immobilisation of the capture antibody (anti-cardiac Troponin T 1C11 antibody) onto a gold surfaced SPR sensor chip, to which a self-assembled monolayer of 11-mercaptoundecanoic acid has been applied. A direct immunoassay for cTnT in buffer was examined and a limit of detection (LOD) of 25 ng ml^{-1} cTnT was achieved. A sandwich immunoassay format was then developed to enhance the sensitivity of the assay. The use of a detection antibody (anti-cardiac Troponin T 7G7 antibody) was shown to successfully amplify the SPR response five-fold, with the LOD improving to 5 ng ml^{-1} cTnT.

The second stage of the project involved examining the extent of non-specific binding of the cTnT and of serum proteins, and investigating how best to minimise and control for it. Non-specific binding of cTnT was eliminated, and serum protein binding was reduced by 93% in 10% serum and 73% in 50% serum. To achieve greater sensitivity, amplification of the signal through the use of detector antibodies conjugated to gold nanoparticles (AuNPs) for the

sandwich assay was investigated. The performance of the cTnT immunosensor sandwich assay in human serum was evaluated using non-modified and AuNP modified detector antibodies. The LOD of the immunosensor in 50% serum was assessed as 5 ng ml^{-1} cTnT for the standard sandwich assay, and 0.5 ng ml^{-1} cTnT when using AuNP conjugated detector antibodies to enhance the sensitivity.

Keywords:

Surface plasmon resonance; human serum; sandwich immunoassay; gold nanoparticle; non-specific binding

ACKNOWLEDGEMENTS

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ACS	Acute coronary syndrome
AMI	Acute myocardial infarction
AST	Aspartamate aminotransferase
BNP	B-natriuretic peptide
BSA	Bovine serum albumin
CK-MB	Creatine kinase – muscle brain
CeVD	Cerebrovascular disease
CRP	C-reactive protein
CT	Computed tomography
cTnI	Cardiac troponin I
cTnT	Cardiac troponin T
CV	Coefficient of variation
CVD	Cardiovascular disease
DNA	Deoxyribonucleic acid
ECG	Electrocardiogram
EDC	1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide
ELISA	Enzyme linked immunosorbent assay
EU	European Union
fM	Femtomolar
GDF-15	Growth differentiation factor 15
HCl	Hydrochloric acid
H-FABP	Heart fatty acid binding protein
hs-cTn	High sensitivity cardiac troponin
IMA	Ischaemia modified albumin
LD	Lactate dehydrogenase
LOD	Low limit of detection
MI	Myocardial infarction
mL	Millilitres
MRI	Magnetic resonance imaging
11-MUDA	11-Mercaptoundecanoic acid
µL	Microlitres

ng	Nanogram
NHS	N-hydroxysuccinimide
nM	Nanomolar
NSB	Non-specific binding
NSTEMI	Non ST-segmented elevated myocardial infarction
NTproBNP	N-terminal pro B natriuretic peptide
PBS	Phosphate buffered saline
PBS/T	Phosphate buffered saline + 0.05% Tween 20
PET	Positron emission tomography
pg	Picogram
POC(T)	Point-of-care (testing)
QCM	Quartz crystal microbalance
RNA	Ribonucleic acid
SAM	Self assembled monolayer
SE	Stress echocardiography
SPE	Screen printed electrode
SPECT	Single photon emission computed tomography
SPR	Surface plasmon resonance
STEMI	ST-segment elevated myocardial infarction
TAT(s)	Turn around time(s)
TIA	Transient ischaemic attack

CHAPTER 1

INTRODUCTION

1 INTRODUCTION

1.1 Cardiovascular Disease

1.1.1 Overview and statistical background

Globally, cardiovascular disease (CVD) is currently the biggest cause of mortality, having taken over from infectious diseases. Approximately 17.3 million or 30% of the world's population dies each year from CVD, and morbidity rates are even higher (WHO, 2011a). Similarly, recent figures from the British Heart Foundation indicate that over 32% of deaths in the UK can be attributed to CVD (Townsend et al., 2012).

In Europe, especially in the EU countries, government education and prevention campaigns have begun to make an impact, and over the last three decades the mortality figures have started to come down (Nichols et al., 2012). Although a downturn has begun in westernised countries, the figures for the developing world have recently been increasing significantly, as incomes improve, leading to more consumption of 'fast food' and adoption of more sedentary lifestyles (WHO, 2011b). These figures are only likely to rise for the next few decades, before similar educational campaigns have an impact. It is estimated that 23.3 million people will die globally each year from CVD by 2030 (Mathers and Loncar, 2006).

Having CVD brings both a social and financial burdens to society. The patient is unable to earn a living, family members may have to give up their own work to become carers, and a disproportionate amount of their income is spent on medication. Similarly it places a large economic burden nationally. In total, based on direct healthcare costs, informal care costs and lost productivity (from mortality and morbidity), in 2009 CVD cost the UK £29 billion (Townsend et al., 2012). In low and middle income countries, medical care costs, premature mortality, etc. can reduce Gross Domestic Product (GDP) by up to 6.77% (WHO, 2011b).

Hence, there is a lot of research being directed into the early detection and accurate diagnosis of cardiovascular diseases, so that globally mortality and

morbidity can be reduced by initiating the most appropriate treatment or preventative intervention as soon as possible. This will save lives, improve the quality of life for those who already have CVD, and help to reduce healthcare costs and the economic burden from CVD.

1.1.2 Different types of cardiovascular disease

CVD refers to a group of many related conditions, which affect medium to large arteries, and consequently can also affect the heart itself. Most CVDs are chronic conditions which increase in severity with time, as arteries become narrowed, and blood flow is restricted. However, acute conditions can arise when a blood vessel suddenly gets blocked (by a thrombus or embolus), leading to heart attacks or strokes, depending on the location of the blockage.

1.1.2.1 Atherosclerosis

Atherosclerosis is a major form of CVD, and contributes significantly to mortality arising from CVD (WHO, 2011a). Atherosclerosis occurs after an injury to the endothelium of the artery wall, or where smooth muscle wall cells are missing. Monocytes can adhere and transmigrate, and smooth muscle cells can also migrate into the intima, where they can accumulate extracellular lipid. The monocytes turn into macrophages, which by engulfing modified lipoproteins then become lipid laden foam cells, as shown in Figure 1.1 (Libby, 2001).

The fatty streaks swell up as they accumulate lipids, calcium and debris, with a covering of fibrous connective tissue, forming what is known as an atheromatous plaque. Plaques build up on artery walls, which narrow the diameter of the arteries, constricting the blood flow through the arteries. This makes it harder for blood to flow through the arteries, and they get damaged easily. If the fibrous cap is thick and stable, it can lead to conditions like angina. However, if the fibrous cap is thin, it can rupture leading to a heart attack (Libby and Theroux, 2005).
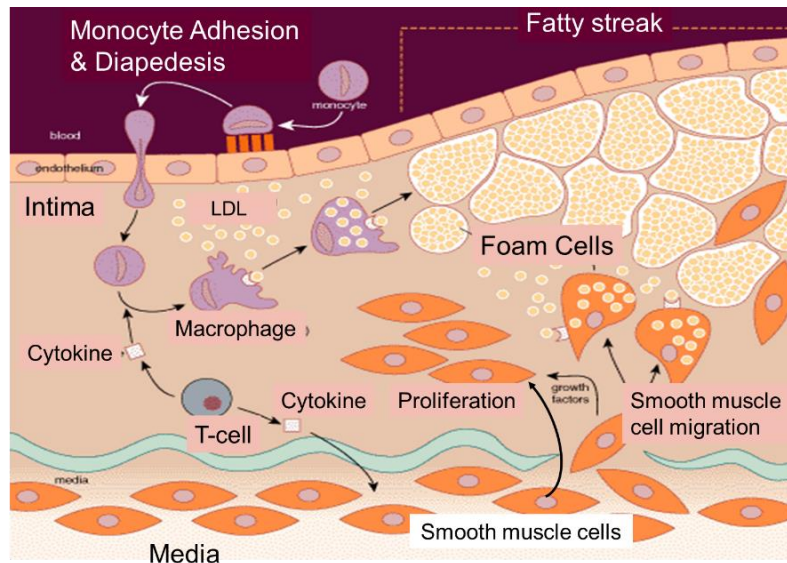
Figure 1.1:   Schematic of the start of the atherosclerotic process.

Illustration reproduced from http://sphweb.bumc.bu.edu/otlt/MPH-Modules/PH/PH709_Heart (accessed 12 Dec 2013).

1.1.2.2 Acute coronary syndrome and myocardial infarction

Acute coronary syndrome (ACS) is the name given to group of conditions which occur when the blood supply to the heart suddenly gets obstructed, such as myocardial infarction (MI) or unstable angina. When blood vessels get damaged or an atheromatous plaque ruptures, it enables coagulant factors to access the thrombogenic lipid core, leading to the formation of a thrombus (blood clot). The thrombus can cause a sudden total occlusion of that artery (Figure 1.2), blocking blood flow to the heart and is known as an acute myocardial infarction (AMI), more commonly called a heart attack. The lack of blood prevents oxygen and nutrients getting to the heart muscle cells leading to their death, resulting in myocardial tissue necrosis, which causes permanent damage to the heart (Libby, 2001).
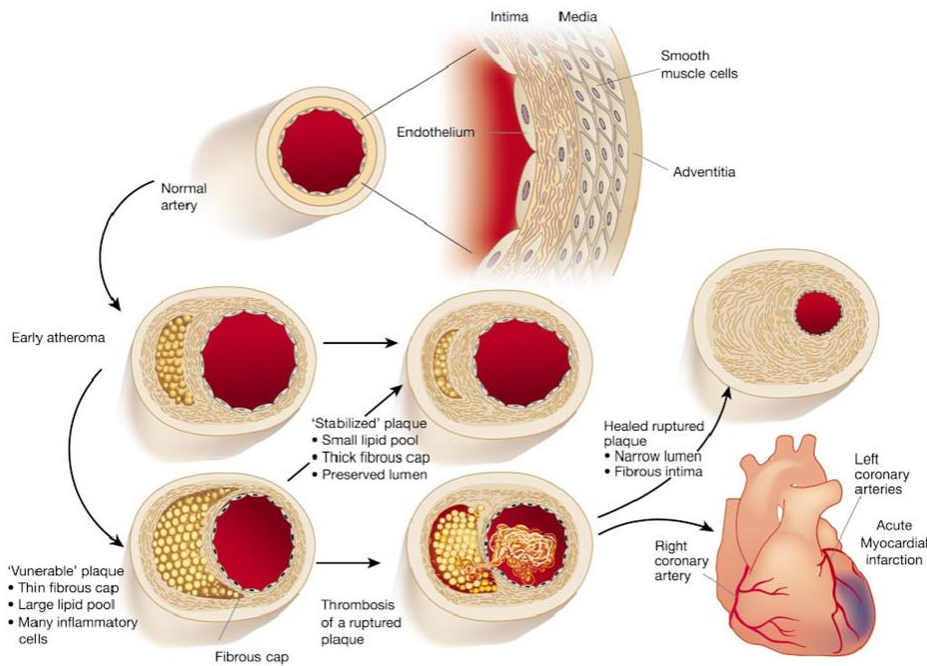
Figure 1.2: **Schematic of Plaque formation leading to narrowed arteries and/or acute myocardial infarction (Libby, 2002).**

1.1.2.3 Angina

As atherosclerosis worsens, the restriction of the blood supply to the muscles of the heart (due to the hardening and narrowing of the arteries) can cause chest pain, known as angina. Angina is usually triggered by events such as physical exertion, stress or an emotional upset, cold weather, or a heavy meal. There are two types of angina; stable and unstable, both of which have similar symptoms of tightness/heaviness in the chest, and pain spreading out to the arms, neck, and jaw, together with breathlessness (Gray et al., 2002). Stable angina usually occurs when the heart has to work harder and insufficient blood is delivered to the heart, but starts to dissipate on resting for a few minutes. Unstable angina is unpredictable, and may occur without a trigger and persist even on resting (Anderson et al., 2007).

1.1.2.4 Cerebrovascular disease and stroke

Another CVD condition is cerebrovascular disease (CeVD) which affects the blood vessels supplying blood to the brain. With CeVD fatty deposits do not

build up in the arteries. However, the blood vessels are affected by increases in blood pressure (hypertension), and endothelial cell injury can cause aggregation of platelets, as they try to repair the damage. Generally the blood vessels get stiffer, thus making them more vulnerable to damage. If these blood vessels either rupture or get blocked, the brain loses its blood and nutrient supply, leading to what is commonly known as stroke. The death of brain cells causes brain damage, which depending on the severity can result in disability or death. Strokes are the third biggest cause of mortality in the UK after coronary heart disease and cancer. They are also the biggest cause of adult disability in the UK (Adamson et al., 2004).

There are two main types of stroke which are differentiated by their causality. Ischaemic strokes are the most common (accounting for over 80% of cases), which is where the blood supply to the brain is cut off due to a blood clot (Roach et al., 2008). The other form is haemorrhagic (also known as cerebral or intracranial haemorrhages), where a blood vessel ruptures due to weakness in the vessel wall, and bleeds into the brain. There is also a third form of stroke known as a transient ischaemic attack (TIA), sometimes referred to as a 'mini-stroke', where the blood flow to the brain is only halted temporarily, so that symptoms disappear (Easton et al., 2009). Although not as damaging, they need to be heeded as a warning that a full blown stroke is likely in the future, and should be treated seriously. Studies have shown that if a person has a TIA, they have a 10% chance of having a full blown stroke in the next four weeks (Truelsen et al., 2006).

1.1.2.5 Myocarditis

Myocarditis is caused by inflammation of the heart muscle, also known as the myocardium (Gray et al., 2002). While a variety of things may cause myocarditis, the most frequent in the UK, is a viral infection from common viruses such as adenovirus or parvovirus. Worldwide it is more likely to be caused by protozoan infections (leading to Chagal's disease) (WHO, 2011a). While myocarditis may present symptoms very similar to ACS, the arteries will not be blocked.

1.2 Biomarkers of Cardiovascular Disease

Initially diagnosis of AMI was carried out by assessing the presenting patient's physical symptoms. Later diagnosis was modified to include assessments by ECGs, monitoring the patient's heart rhythms via the heart's electrical activity. Biomarker assays were then developed to help emergency department physicians verify retrospectively if a patient may have had an AMI (by confirming myocardial necrosis) even though an ECG has been inconclusive (Lott and Stang, 1980; Mair et al., 1992).

The use of biomarkers in helping with the diagnosis of CVD, was one of the earliest successful uses of biomarkers in medicine. The first molecules to be used in this way were enzymes. The earliest enzyme biomarker to be used in cardiology was serum glutamic oxacetic transaminase (LaDue et al., 1954; Karmen et al., 1954), which was later re-named as aspartamate amino-transferase (AST). This team's further research into other enzymes which could potentially monitor myocardial necrosis, resulted in the discovery of lactate dehydrogenase (LD) shortly afterwards (Wroblewski et al., 1955). However, neither of these two enzymes were specific for cardiac tissues, as they were later found to be present in liver and skeletal tissues amongst others.

Further research into biomarker enzymes identified creatine kinase, especially the isozyme creatine kinase – muscle-brain (CK-MB) as being more cardiac specific. It took several years until a suitable enzyme assay could be developed for the quantitation of CK-MB (Rosalki, 1967). Once developed, this assay became an important tool in AMI diagnosis for the next couple of decades. In addition to CK-MB being more cardiac specific, it could help to confirm within 24 hours of their admittance, that a patient had undergone an AMI, rather than the previous 2 to 3 days when measuring AST and LD enzymes. The demand for improved CK-MB analysis, with quicker turn around times (TATs) and more cardiac specificity increased during 1980s, due to new treatments for AMI becoming available such as streptokinase in 1982 and tissue plasminogen activator in 1987 (Chesebro et al., 1987; Crabbe and Cloninger, 1987), and also due to an increase in false positive results (Bhayana and Henderson, 1995).

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The development of a two-site specific monoclonal antibody for CK-MB allowed much more specific and sensitive sandwich immunoassays with TATs of less than 15 minutes to be developed which are still commonly in use today (Wu et al., 1985). This period of time coincided with clinical research showing that the earlier medical intervention occurred after an AMI, the less myocardial damage was incurred, and the better the outcome for the patient with lower rates of mortality and morbidity (Collins et al., 1993).

Following improvements in protein identification and purification, the search for more specific cardiac biomarkers which wouldn't give false positives was renewed. The next major discovery in cardiac markers was the cardiac troponins, (cardiac troponin I (cTnI), by Cummins et al., in 1987, and cardiac troponin T (cTnT) by Katus et al., in 1989, which were very specific for cardiac tissue damage. There was initially reluctance to use troponins for diagnosis, as they appeared to produce a higher rate of false positives, based on the WHO criteria at the time. Over the next decade over a hundred clinical studies were carried out, and the cumulative data proved that both cTnI and cTnT were equally effective for use in diagnosis of AMI. The data obtained showed that troponins also had a significant and important role in the prognosis of ACS patients and their risk stratification. Raised troponin levels in ACS patients could influence the therapy or intervention they received, to reduce their risk of suffering major cardiac events in the future (Dolci and Panteghini, 2006).

As more positive results emerged from the research, there was a consensus to make cardiac troponins the "gold standard" biomarker in AMI diagnosis, which was approved in 2000 (ESC/ACC Global MI task force, 2000). The definition of AMI and the criteria to diagnosis it, was again refined in 2007 and presented as a universal definition (Thygesen et al., 2007).

Subsequently, continued research into troponin measurement has significantly improved the sensitivity achievable, leading to high sensitivity cardiac troponin (hs-cTn) assays which have the potential to give an earlier indication of AMI in the initial phase after the onset of symptoms. Thus, the new data and insights

have been used to prepare a new third universal definition of MI, as described in Section 1.2.3 (Thygesen et al., 2012).

However, the search continues to find alternative biomarkers which may give an earlier reliable indication of a MI event (McCann et al., 2008; Lin, 2012; Aldous et al., 2013). By studying the physiological pathway of atherosclerosis and MI and looking at the earlier stages, biomarkers were identified which can be grouped into markers of inflammation, plaque destabilisation, plaque rupture, myocardial necrosis and ventricular remodelling, as detailed in Section 1.2.6.4.

The alternative early biomarkers which have been researched most are C-reactive protein (CRP), ischaemia modified protein (IMA), heart fatty acid binding protein (H-FABP) (Ramasamy, 2011). Raised levels in the blood stream can be measured from 1.5 to 2 hours post-infarction, much earlier than the troponins (Kehl et al., 2012). While these newer biomarkers are found to be poor at giving a definitive diagnosis of MI on their own, they can be useful for prognosis and risk stratification. Thus, the trend now is to measure multiple biomarkers to build up a profile of the disease (Cameron et al., 2007; Xu et al., 2010, Fakanya et al., 2014).

1.2.1 Myoglobin

Myoglobin was the first non-enzymatic protein to be identified as a cardiac biomarker (Figure 1.3), when Kagen et al., (1975) observed that raised concentrations correlated with myocardial infarction. Myoglobin is a small (17 kDa) protein molecule, which is the earliest routine biomarker released after damage occurs to myocardial muscle. Concentrations are usually measurable 1.5 hours after an AMI event, peak between 6 and 12 hours, and then return to normal levels over the next 24 hours (Mair et al., 1992). This early increase in concentration, led to myoglobin being included in multi-marker panels with other cardiac markers such as CK-MB and troponins, to help with early diagnosis of AMI (Newby et al., 2001; McCord, 2003; Yamamoto et al., 2004).
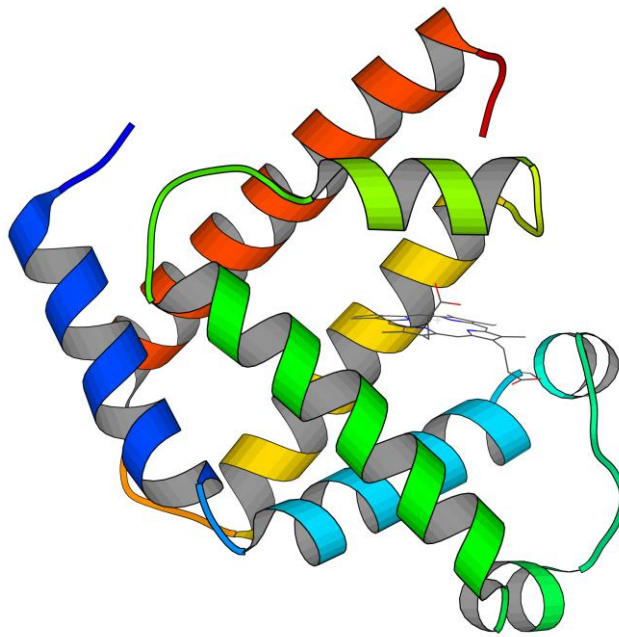
Figure 1.3:   Tertiary structure of myoglobin.

Reproduced from xray.bmc.uu.se/lars/Courses/BAllb/databases_09/01.html (accessed 07 Jan 2014).

However, there are several reasons why myoglobin cannot be used on its own for AMI diagnosis. Myoglobin is not a cardiac specific biomarker, due to both skeletal and cardiac myoglobin having the same amino acid sequence. Thus skeletal muscle injury or renal disease will also increase myoglobin concentrations (Azzazy and Christenson, 2002). Due to its short half-life myoglobin does not remain in the circulation for long, so a diagnosis of AMI could be missed if the patient presents to the emergency department 24 hours after the onset of symptoms.

1.2.2 Creatine Kinase – Muscle Brain (CK-MB)

Creatine Kinase – Muscle Brain (CK-MB) was identified as the first "true" cardiac biomarker for an AMI, as its formation was proportional to the amount of myocardial damage (Jaffe, 1991). During the 1970s, 80s and mid 90s serial measurements of CK-MB were used as the "gold standard" for diagnosis of AMI (Saenger and Jaffe, 2008; Kehl et al., 2012). Serial measurements were used

as CK-MB concentrations doubled 5 to 6 hours after an infarction, and then peaked between 12 and 24 hours (Yang, 2006; McDonnell et al., 2009).

However, a major disadvantage in using CK-MB, was that it was not particularly cardiac specific, and levels could easily become raised after skeletal muscle tissue damage, renal disease, or even just exertion such as marathon running (Shave et al., 2010). Improvements to assays were made as immunoassay knowledge increased, but the advent of more sensitive assays caused a lot of false positive results (up to 20%), so CK-MB was superseded by cardiac troponin as the "go to" cardiac biomarker (Saenger and Jaffe, 2008; Dolci and Panteghini, 2006).

1.2.3 Troponins

Troponin I and Troponin T are regulatory proteins which are part of a complex (also including Troponin C and Binary I-C complex), responsible for muscle contraction (Figure 1.4). The complex is located within the myocyte contractile apparatus which regulates the calcium mediated contraction of myosin and actin filaments (Daubert and Jeremias, 2010). Specific isoforms of these proteins are expressed in certain tissues, namely skeletal and cardiac muscle (Gaze and Collinson, 2008). This has enabled the development of very specific and sensitive assays for these two cardiac troponins (cTnI and cTnT), since antibodies can be generated that do not react with the skeletal isoforms (Melanson et al, 2007).

Small amounts of free cardiac troponins (approximately 7% of cTnT and 4% of cTnI) are present in the cytoplasm, while the rest is bound (Katus et al., 1991; Higgins and Higgins, 2003). During a MI, the cell membranes of cardiac myocytes are damaged, which releases both the cytoplasmic troponins, and the T-I-C complex.
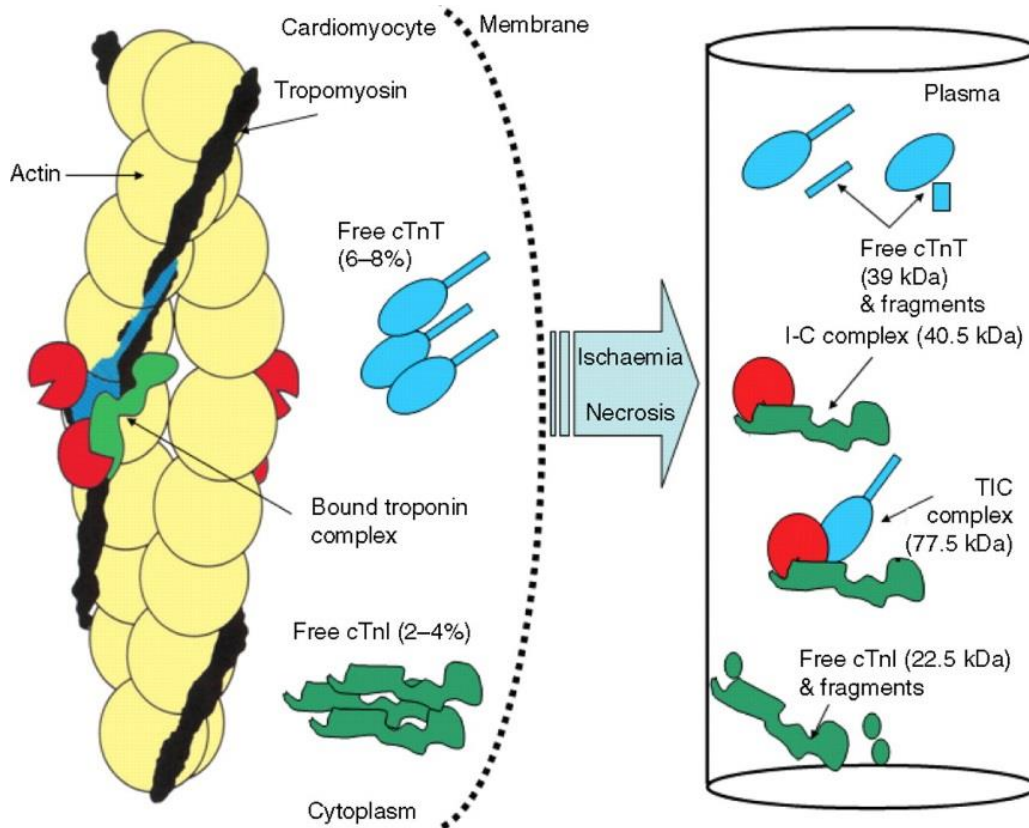
Figure 1.4:   Structure of the cardiac troponin complex, and troponin forms.

Reproduced from www.abs.sage.pub.com/45/4/349 Fig 1. (Accessed 20 Dec 2013)

It takes 2 to 4 hours for necrosis to start; hence troponin levels are only measurable after this minimum period of time post infarction (Thygesen et al., 2007). Through proteolytic degradation the complex is broken down in the blood, releasing further troponin units, which contributes to the continued rise of troponin concentrations, which peak after 12 to 24 hours (Daubert and Jeremias, 2010; Morrow et al., 2007). Troponin concentrations stay raised above normal levels for about 4 to 10 days (Figure 1.5) which is useful for diagnosing patients with late presentation of AMI after onset of symptoms (Morrow et al., 2007).
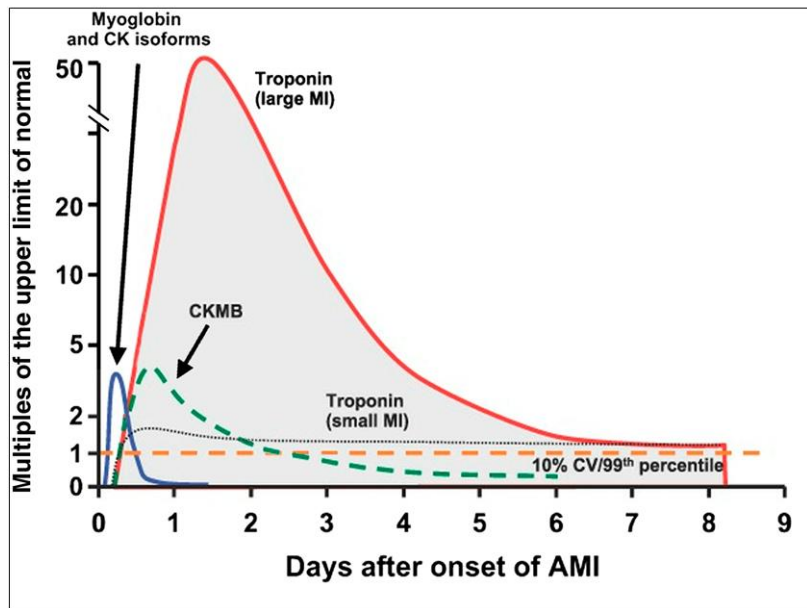
Figure 1.5: Cardiac marker time profile after an AMI event (Anderson et al., 2007).

The accurate diagnosis of AMI has developed over the years as more information about cardiac biomarkers has become available, and cardiac troponins now play a central role. A universal definition of MI has recently been agreed with the following criteria;

"*Detection of a rise and/or fall of cardiac biomarker values [preferably cardiac troponins (cTn)] with at least one value above the 99th percentile upper reference limit (URL) and with at least one of the following:*

- *Symptoms of ischaemia.*

- *New or presumed new significant ST segment-T wave (ST-T) changes or new left bundle branch block (LBBB).*

- *Development of pathological Q waves in the ECG.*

- *Imaging evidence of new loss of viable myocardium or new regional wall motion abnormality.*

- *Identification of an intracoronary thrombus by angiography or autopsy.*" (Thygesen et al., 2012).

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As well as AMI, raised troponin levels can be used to diagnose a number of other cardiac conditions such as; severe pulmonary embolism, myocarditis and cardiotoxicity, but in these cases levels will be at a much lower concentration, although present for a prolonged period of time (Hamm et al., 2002; Meyer et al., 2012).

1.2.3.1 Cardiac Troponin T (cTnT)

Troponin T is a binding protein subunit of the troponin complex which attaches troponin complex to tropomysin and regulates its interaction with thin filaments induced allosteric signals from calcium ions (Jin and Chong, 2010). The patenting of the cTnT antibodies by Roche, standardised the assay (based on the stable portion of the molecule), and allowed very low cut-off concentrations (due to less variability).

Whilst the extra sensitivity was an advantage, allowing AMI to be detected sooner, it also caused clinicians a lot of confusion as to how to treat patients with raised but low levels of cTnT, in the absence of any obvious ischaemic heart disease (Jaffe, 2012), which applies to cTnI as well. An issue with the improved cTnT assay sensitivity which became more apparent, was slightly reduced specificity (possibly from cross-reactivity) as cTnT could now be detected in patients with major skeletal muscle disease (Jaffe et al., 2011). However, a study by de Antonio et al., (2013) using a head to head comparison has shown that both cTnT and cTnI were equally good for diagnosis of heart failure patients, but cTnT being better at predicting a higher proportion of patients that would die.

1.2.3.2 Cardiac Troponin I (cTnI)

Troponin I inhibits ATP-ase activity of acto-myosin, modulating the interaction of actin and myosin. Troponin I is expressed as both skeletal and cardiac isoforms. Compared with cTnT, the cardiac isoform cTnI has so far proven to be very specific to cardiac muscle, and is not found in any skeletal muscle tissues (McDonnell et al., 2009). Troponin I can exist in bound forms (the three subunit complex TIC, the two subunit I-C complex, or in a complex with troponin T and or troponin C), and in the free subunit form (where it can be oxidised,

reduced or phosphorylated) as shown in Figure 1.4 (Katrukha et al., 1998, 1999). The different cTnI species are cleared from the body at different rates. This can lead to variability in values being reported for the same sample by different assays (Table 1.1), due to the epitope specificity of the antibody (Katrukha et al., 1999; Apple and Collinson, 2012). The variability in results observed between different commercial assays, makes it difficult (inaccurate) to compare results between clinical studies (Tate and Panteghini, 2011; Apple et al., 2012). This has led to calls for agreeing to use a specific reference material, to standardise commercial assays for comparison (Tate and Panteghini, 2011).

Table 1.1: Different epitopes used by commercial cTnI assays (Wu, 2010).

Company/platform/assay (generation)	Epitopes recognized by antibodies
Abbott AxSYM ADV (2nd)	C 87-91, 41-49; D 24-40
Abbott Architect	C 87-91, 24-40: D: 41-49
Abbott i-STAT	C: 41-49, 88-91; D: 28-39, 62-78
Beckman Access Accu (2nd)	C; 41-49; D: 24-40
bioMerieux Vidas Ultra (2nd)	C: 41-49, 22-29; D: 87-91, 7B9
Innotrac Aio!	C: 41-49,190-196; D: 137-149
Inverness Biosite Triage	C: NA; D: 27-40
Mitsubishi Chemical PATHFAST	C: 41-49; D:71-116, 163-209
Ortho Vitros ECi ES (2nd)	C 24-40, 41-49; D 87-91
Radiometer AQT90	C; 41-49, 190-196; D: 137-149
Response Biomedical RAMP	C: 85-92; D: 26-38
Siemens Centaur Ultra (2nd)	C: 41-49, 87-91; D: 27-40
Siemens Dimension RxL (2nd), Stratus, Vista	C: 27-32; D: 41-56
Siemens Immulite 2500 and 100	C: 87-91:D: 27-40
Tosoh AIA II (2nd)	C: 41-49; D: 87-91

1.2.4 C - reactive protein

C-reactive protein (CRP) is a non-specific biomarker of inflammation, first discovered in 1930 by Tillet and Francis. The CRP molecule is a short pentraxin molecule made up of five 23 kDa subunits (Ridker, 2003). It is produced mostly in the liver, and formed on stimulation from inflammatory cytokines (mainly interleukin-6). Increased CRP levels are detectable 6 hours

after stimulation, they reach peak concentrations within 48 hours, and have a half-life of approximately 19 hours (Pepys and Hirschfeld, 2003). Studies of myocardial damage identified raised levels of CRP after MI, which led to a lot of interest in trying to use them for early prediction of MI events. CRP was shown to be a better predictor of cardiovascular risk than cholesterol (Ridker et al., 2003). With the advent of high sensitivity CRP (hs-CRP) assays, research has shown that increased baseline concentrations of hs-CRP are strongly linked with death in patients with ACS that initially survived an AMI (Scirica et al., 2007; Suleiman et al., 2006).

1.2.5 Brain natriuretic peptide (BNP)

Atrial natriuretic peptides are a group of peptides which are synthesized and secreted by the ventricular myocardium. They are involved in endocrine regulation, linking the heart with the renal system. During heart failure they lower the pressure in the lungs, increase renal flow and inhibit the rennin-angiotensin-aldosterone system (Suzuki et al., 2001). When the ventricular myocardial wall experiences excess pressure, it stimulates the production of the pre-hormone proBNP, which is a 108 amino acid peptide. This is cleaved into two smaller peptides; B natriuretic peptide (the active 32 amino acid C-terminal end) and NTproBNP (the longer 76 amino acid N-terminal fragment), which is inactive (Newton et al., 2009). Either fragment can be used as a cardiac biomarker, as both are found to be raised (up to 1000 fold) in patients that have left ventricular dysfunction (Suzuki et al., 2001). Concentrations of NTproBNP are usually found to be 5 to 6 times higher than that of BNP, due to its longer half-life, and so are often measured preferentially, as concentration levels are easier to measure (Weber and Hamm, 2006).

BNP and NTproBNP are used to aid the diagnosis of ventricular overload, but cannot be used solely to diagnose heart failure or ACS, as raised levels can be present with conditions such pulmonary embolism or renal failure. However, they are excellent markers for risk stratification, being good at predicting mortality and morbidity post AMI (Richards et al., 1999). Often patients can present at emergency departments with symptoms which may be caused by

heart failure but are not specific to it, and the results of a physical exam and ECG are inconclusive. Studies have shown that in these cases BNP and NTproBNP are useful markers for determining quickly and accurately if a patient's breathlessness is due to heart failure, or other causes, and so enabling the correct therapy to be started promptly. This is important due to the treatment used for heart failure being hazardous for patients with chronic obstructive pulmonary disorder (COPD), and vice versa (Mueller et al., 2004; Newton et al., 2009).

1.2.6 Emerging Biomarkers

1.2.6.1 Ischaemia Modified Albumin

Ischaemia modified albumin (IMA) is a molecule which has been put forward as a potential cardiac biomarker, as levels have been shown to be raised following ischaemic injury, both myocardial and cerebrovascular (Bhagavan et al., 2003; Herrison et al., 2010). Plasma albumin can bind divalent ions to its N-terminal end. During ischaemia the N-terminal end of human serum albumin is modified, which affects its capacity to bind cobalt. A colorimetric cobalt binding assay is then used to measure the concentration of IMA (Bar-Or et al., 2001).

There has been interest in measuring serum IMA levels, as IMA may provide early specific diagnostic information about the presence or severity of coronary artery disease, where ischaemia has occurred, but not yet progressed to myocardial necrosis (Kazanis et al., 2009). Generally the results from meta analyses of clinical studies have been mixed, and it is suggested that IMA should be used with other markers (Ramasamy, 2011).

1.2.6.2 Heart type Fatty Acid Binding Protein

Heart type fatty acid binding protein (H-FABP) is a small cytostolic protein which is important in the transport of long chain fatty acids in cardiac myocytes, where it is predominantly found (although very low concentrations can also be found in skeletal muscle, kidney and brain tissue). After cell membrane damage from ischaemia, H-FABP is released into extra cellular space, and can be observed just 1.5 hours after damage (Figure 1.6), so giving an indication of ischaemia

before definitive detection of myocardial necrosis. The concentration of H-FABP peaks between 5 to 6 hours, and returns to normal by 36 hours after the event (Glatz et al., 1994).
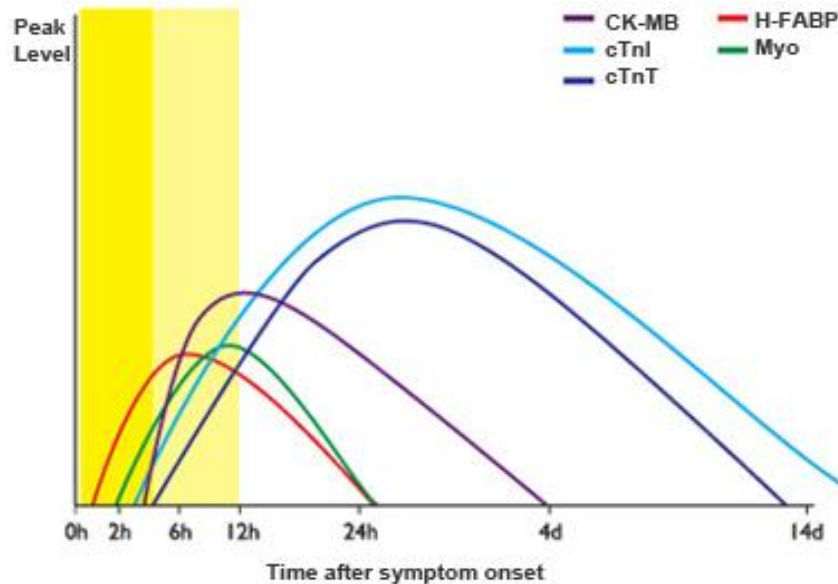
Figure 1.6:   Time profile of H-FABP and other cardiac markers.

Reproduced from www.randox.com/H-FABP_assay/php (accessed on 18 Dec 2013)

Due to its lack of specificity and overall sensitivity, many studies have examined its use in combination with other cardiac biomarkers (Okamoto et al., 2000; Ishii et al., 2005; McCann et al., 2008). The potential of H-FABP to be used for risk stratification and prognosis has also been investigated in several studies, with promising results. Higher concentrations of H-FABP correlated closely with a higher risk of mortality or adverse cardiac events in the long term, independently of other biomarkers (Erlikh et al., 2005; Ishii et al., 2005; Kilcullen 2007; Viswanathan et al.,2010). However, more research needs to be done to establish reference values and variation amongst different populations (e.g. gender, age, renal disease).

1.2.6.3 Growth-differentiation Factor 15 (GDF-15)

GDF-15 is a cytokine which belongs to the transforming growth factor-β family. It is present at low levels in healthy individuals, but increases under conditions

of inflammation, tissue damage and oxidative stress, thus concentrations are considerably raised in CVD (Wollert and Kempf, 2012). However, although expression of GDF increases within the heart after infarction, it is mostly produced outside of the myocardium. Thus, GDF-15 is currently of great interest, as it may link information obtained from cardiac and non-cardiac disease pathways. Recent studies have shown that even though it is not cardiac specific, it is an independent risk and prognostic biomarker in patients with AMI and ACS (Kempf et al., 2007; Norozi et al., 2011; Widera et al., 2012).

1.2.6.4 Other investigated potential cardiac biomarkers

As mentioned at the end of the introduction to Section 1.2, the search for new markers of AMI has examined the pathophysiology of atherosclerosis and ischemia and identified possible candidates, at each stage of the process, as depicted in Figure 1.7. Over 60 biomarkers have been or currently are being evaluated for their efficacy in helping to diagnose AMI earlier, and/or for risk stratification and prognosis, to enable better monitoring and treatment of cardiovascular disease (Freiss and Stark, 2009; Kehl, 2012; Van Kimmenade and Januzzi, 2012).

Plaque formation	Plaque rupture	Thrombosis	Ischaemia	Necrosis	Heart failure
LDL CRP IL-6 TNF LP-PLA2 Galectin-3	MMP MPO ICAM VCAM PAPPA	sCD40L PIGF D-Dimer	IMA uFFA Choline H-FABP	cTnT cTnI CK-MB Myoglobin Copeptin	BNP NT-BNP Copeptin GDF-15 sST2
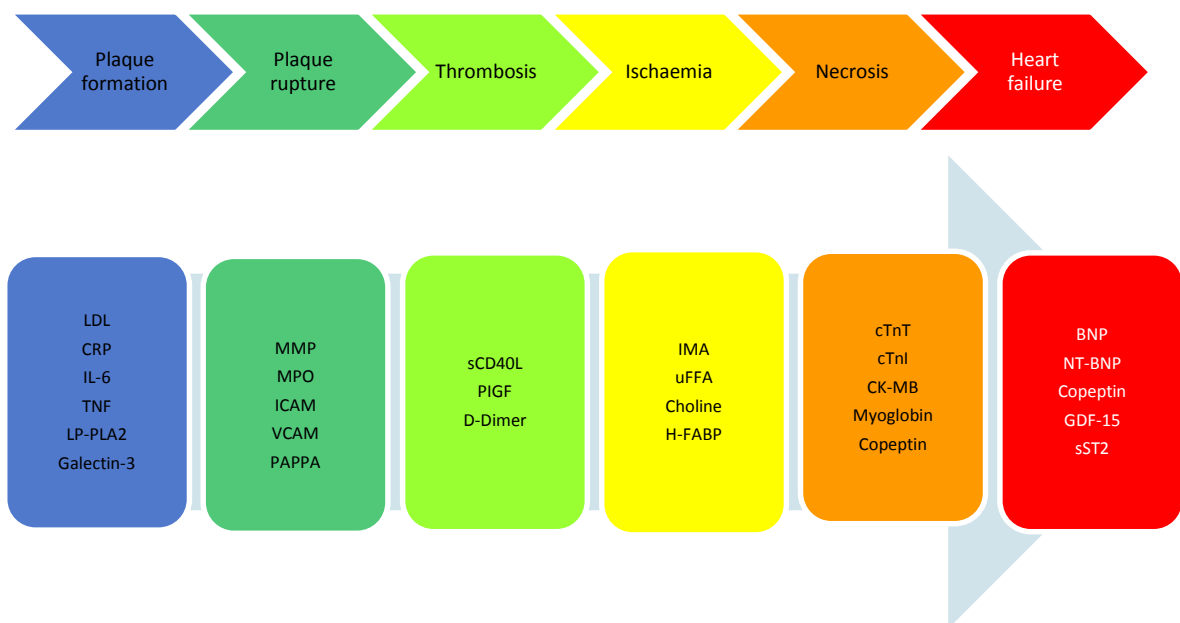
Figure 1.7: Schematic of pathophysiology of acute coronary syndrome (ACS) and corresponding markers.

With so many studies going on, each slightly different in format and objectives, it is difficult to critically compare and evaluate the data produced. Morrow and de Lemos (2007) have put forward a benchmarking system to help standardise the approach taken to evaluating a new marker, based on a three question approach.

"1.   Can the clinician measure the marker accurately?

2.   Does the biomarker add new information?

3.   Will the biomarker help the clinician to manage patients?"

(Morrow and de Lemos, 2007)

Using the suggested approach will help provide a solid basis for the evaluation of potential new cardiac markers, which will be of true benefit to the diagnosis, prognosis and treatment of CVD.

1.3 Diagnosis of cardiovascular disease

1.3.1 Clinical diagnosis

The different cardiovascular diseases can be diagnosed and confirmed in many ways using a variety of techniques. However, it is essential to diagnose an AMI as soon as possible, so that appropriate treatment (e.g. reperfusion) can be started immediately. The goal is to minimise the amount of myocardial muscle damage sustained (the adage in cardiology being 'time is muscle'), as the time window to prevent excessive damage is only 1 hour (Collins et al., 1993).

To diagnose AMI, the original ESC/ACC criteria were that the patient has to present two out of three of the following;

   i)   chest pain and other physical symptoms of ischaemia such a shortness of breath.

   ii) a raised ST segment and/or abnormal Q wave in the electrocardiogram (ECG).

   iii) significantly raised levels of cardiac biomarkers (especially cardiac troponins).

(Luepker et al., 2003).

The definition of AMI and criteria used has been continually refined, as outlined in more detail in Section 1.2.3 (Thygesen et al., 2012). Further detailed investigation and assessment can be carried out by using techniques such as; echocardiograms, biopsies, MRI or CT scans, and clinical chemistry tests. These can help to give a more specific diagnosis and also monitor the progress of the patient's condition.

1.3.2 Electrocardiogram

The heart is a muscular pump made up of two upper chambers (atria) and two lower chambers (ventricles), which working together, pump blood around the body. The electrical activity produced during this process can be monitored using between 6 to 12 electrodes attached to the skin surface. The minuscule changes in electrical activity are amplified, recorded, and translated into lines on a graph by a device called an electrocardiogram (often known as an ECG or EKG). The patterns of spikes and dips (known as waves) can be interpreted to provide detailed information about how the heart is functioning, any underlying medical conditions or abnormalities and their locations. Figure 1.8 shows a simplified ECG trace.
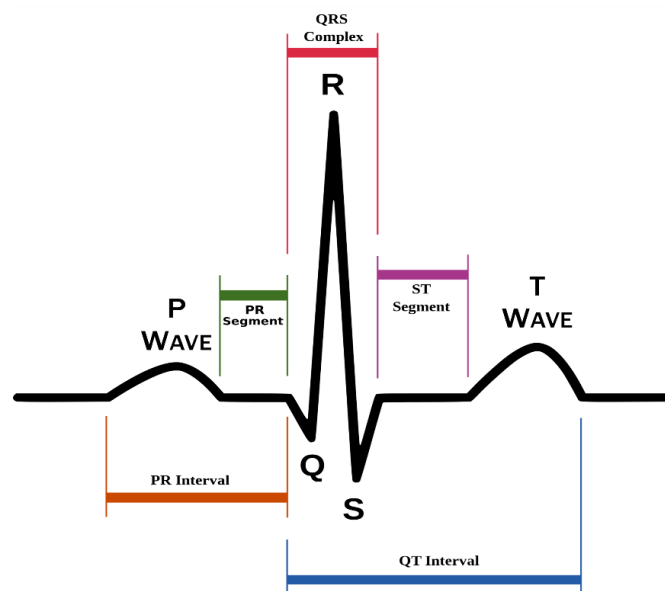
Figure 1.8: Diagram to show trace of an electrocardiogram (ECG).

Reproduced from www.web-books.com/eLibrary/ON/B0/B7/06MLongQT.html

The ST segment of an ECG is of particular importance to the diagnosis of AMI and ACS, and the subsequent treatment of the patient. If the ST segment is elevated from the isoelectric baseline, the heart attack will be classified as a ST-segment elevated myocardial infarction (STEMI). In STEMI, the artery is completely blocked by a clot, and the total absence of blood flow causes the full thickness of the muscle supplied by that artery, to be damaged. Treatment for a STEMI is to return the blood flow (reperfusion) as soon as possible, either pharmacologically by clot-busting drugs (e.g. streptokinase or tissue plasminogen activator), or mechanically by inflating a balloon inside the artery to open it up (Ramasamy, 2011; Welch et al., 2012).

If the ST segment is not elevated, then it is classified as a "non ST-segment elevated myocardial infarction" (NSTEMI). The artery will only be partially blocked, thus the treatment is to use blood thinning drugs to prevent the clot from growing larger. Further investigations can be carried out, and appropriate treatment can be chosen to prevent further long term damage to the heart, e.g. a stent, further drug therapy, or coronary artery bypass grafting (Ramasamy, 2011).

1.3.3 Imaging techniques

1.3.3.1 Overview

A wide variety of imaging techniques play an important role in the diagnosis, management and prevention of CVD. Chest X-rays and angiography were the earliest (1920s) imaging techniques used in cardiology (Linton, 1995). Since then, new techniques have been developed and continuously improved to provide better ways of visualising the heart and its function. Imaging techniques can be divided into two categories; invasive and non-invasive, the latter of which can be further sub-divided into anatomical and functional imaging techniques (Roobottom et al., 2011). Anatomical techniques (e.g. MRI, CT, non-invasive angiography) provide images of the heart and coronary tree, whereas functional techniques (e.g. MRI, stress echocardiography, nuclear cardiology) give images of perfusion under stress and the haemodynamic

consequences of coronary artery disease (Schuijf et al., 2005; Mastouri et al., 2010).

1.3.3.2 X-rays

Chest X-rays can be used to provide useful basic information about the heart such as; is the heart enlarged? is there fluid accumulating around the heart or in the lungs? is there an aneurysm? are valves becoming calcified?, or is a tumour is present? (Gray et al., 2002). X-rays are generally not used as a first line of diagnosis in CVD for several reasons; more cardiac specific and detailed information can be obtained from other imaging techniques, they can be difficult to interpret unless a clinician is very experienced and patients should avoid unnecessary exposure to radiation.

1.3.3.3 Echocardiography

Echocardiography is a very widely used, relatively low-cost, non-invasive imaging technique based on ultrasound, where sound waves are used to build up a sonogram image of the heart. Different forms of ultrasound are available; 2D, 3D, 4D (moving 3D pictures) and Doppler (Van Den Bosch et al., 2005; Steeds, 2011). Echocardiography can directly provide information on the shape and size of the heart, aneurysms, any tissue damage present (necrosis), and the pumping capacity. Indirectly, cardiac output and diastolic function information can be estimated. Doppler ultrasound can be used to accurately evaluate blood flow through the whole heart and also visualise if any blood is leaking from the valves (Hoskins, 1999; McLean et al., 2009). In stress echocardiography (SE) ultrasound scans are performed with the patient's heart first at rest, and then under stress. The stress is achieved by the patient undergoing exercise on a treadmill, or is induced with drugs such as dobutamine or adenosine (Elhendy et al., 2002). SE is often used to investigate chest pain to assess if a patient has coronary heart disease with arteries being blocked or partially obstructed, or has cardiomyopathy (Chong and Langer, 2000).

1.3.3.4 Computed tomography (CT)

Development of modern tomography started in the 1970s, once the processing power of computers increased sufficiently. A computed tomography scan (also known as a CAT scan), is generated by taking multiple 2D radiographic (X-ray) images around a single axis of rotation, and so building up a 3D picture. There have been further improvements and variations on the technique, leading to the development of positron emission tomography (PET) scans and single photon emission computed tomography (SPECT).

SPECT scans are increasingly used in diagnosis of CVD. A radioisotope, or a radioligand (designed to bind to specific areas of the body that require imaging), is injected into the patient's blood stream, which is known as myocardial perfusion scintigraphy. Gamma rays are then used for the SPECT scan imaging, which shows all the blood vessels. SPECT scanning exposes a patient to much less intense radiation, than they would receive from an X-ray (Notghi and Low, 2011).

1.3.3.5 Magnetic Resonance Imaging (MRI)

Cardiac MRI is an imaging technique which is becoming very popular, due to the comprehensiveness of the information it provides. Information that is generated can include; ventricular function, rest and first pass perfusion, cardiac wall motion, and cardiac tissue morphology (Schuijf, 2005). There are many variations available, but the fundamental technique is based on applying magnetic fields which cause hydrogen protons in the body (which spin with randomly aligned axes) to align their axes. A radiowave frequency is then applied causing the hydrogen nuclei to resonate. When the radiowaves are switched off, the nuclei relax and emit energy (also as a radiowave), which is measured. The radio waves are applied as sets of pulsed frequencies, and the resulting emitted signals (which are different for diverse types of tissue, fat and blood) are used to build up cross-sectional images (Linton, 1995; Berger, 2002).

Advantages of using MRI are that bones do not obstruct the images, but the disadvantages are that the equipment is very expensive, needs highly trained

staff to use it accurately, and that it cannot be used on patients who have pacemakers, or other metal objects (clips or valves) fitted (Berger, 2002).

1.3.3.6 Angiography

Modern angiography was first developed in the 1920s by Dr E Moniz, and it is still considered the gold standard technique for investigating blood vessels (Linton, 1995). Angiography is deemed an invasive technique as a catheter is usually inserted via an incision into a groin or forearm artery, and guided up to the heart. Once in position, an X-ray opaque contrast dye (usually iodine based) is released. As the blood circulates, X-ray images (using fluoroscopy) are taken at a rate of 15 to 30 per second, to give a real-time moving image of blood flow through the arteries, veins and heart structures. This enables narrowed or blocked vessels to be identified, and also gives information on leaking heart valves and cardiac muscle abnormalities (Gray et al., 2002).

1.3.4 Auto Immunoassay Analysers

Autoanalysers were developed in the 1950s to enable automated routine biochemical testing of diagnostic markers like cholesterol, phosphate and protein in hospital laboratories. A single sample was sent through a variety of tests within a single large machine (rather than technicians conducting all the individual tests), reducing the amount of labour required and also the errors incurred in processing large volumes of samples (Allinson, 2011).

Yarow and Berson (1959) reported the first use of an immunoassay in their work using a globulin (radioisotopically labelled with iodine) to measure insulin, the technique becoming known as radioimmunoassay. Further research during the next decade, led to the development of immunoassays where antibodies were labelled with enzymes ((ELISA) Engvall and Pearlman, 1971, (EIA) Van Weeman and Schuurs, 1971), which reduced exposure to radioactivity.

The use of immunoassays was quickly adopted, and became established as a standard method of quantifying various diagnostic proteins such as cardiac biomarkers. With sample numbers increasing rapidly, autoanalyzers were developed which could perform the multiple steps required for an immunoassay

(adding all reagents, washing, etc.), and enable several different immunoassays to be run for one sample. Using these analysers between 50 to 150 individual samples (and up to 4000 individual tests) can be processed per hour (Allinson, 2011). However, they do have their disadvantages, which has led to the development of smaller sized, more specific instruments, as explained below.

1.3.5 Point-of-care devices

Automated immunoassay analysers together with the related requirements of pneumatic tube delivery systems, trained staff and the associated capital investment are not available in every laboratory. Thus, achieving the National Academy of Clinical Biochemistry guidelines recommendation of TATs of less than 1 hour for cardiac biomarker analysis is difficult (Christenson and Azzazy, 2009). This has led to a large increase in the use of point-of-care testing (POCT) devices. These are often specialised more compact immunoassay analysers, or lateral flow immunoassay based devices.

The aim of POCT devices is to measure concentrations of cardiac markers and report the results quickly, whilst maintaining the quality of the data, in less than an hour, and ideally less than 30 minutes. The characteristics required for POCT devices (apart from fast analysis) are;

- Results generated need to agree with those obtained from central laboratory autoanalysers

- Easy to use, with minimal technical expertise

- Use whole blood directly (no pre-treatment of sample)

- Low maintenance or disposable

- Low cost

- Interface directly with the patient's electronic medical records (to reduce reporting errors).

POCT devices fall into two main categories; hand-held analysers and small bench top analysers, with the latter being the more ubiquitous. A summary of

the different analyzers and POC devices for troponin assays are presented in Table 1.2, comparing their limits of detection.

Whichever type of POCT device is used, they have been shown to have the following benefits; reducing TATs to less than 30 minutes, reducing the length of the patient's stay in the emergency department, reducing costs, and increasing patient satisfaction (Lee-Lewandrowski et al., 2003; Apple et al., 2006; Singer et al., 2005).

Table 1.2: Comparison of Limits of Detection and 99th Percentile values for high sensitivity, sensitive contemporary and POC Troponin assays (Apple et al., 2012).

Manufacturer · analyzer · assay	No. of results	LoD, ng/L	Measurable values >LoD, %	99th percentile of all study participants (90% CI), ng/L
High sensitivity				
Abbott · ARCHITECT *i*2000$_{SR}$ STAT · hs-cTnI	524	1.2	96	23 (16–63)
Beckman · Access 2 · hs-cTnI	524	2.5	80	32 (22–69)
Roche · Cobas e601 · hs-cTnT	524	5	25	15 (13–28)
Siemens · Dimension Vista · hs-cTnI	503	0.5	86	58 (34–125)
Singulex · Erenna · hs-cTnI	524	0.09	100	40 (25–215)
Sensitive contemporary				
Abbott · ARCHITECT *i*2000$_{SR}$ STAT · cTnI	524	9	2	13 (<9–23)
Abbott · AxSYM · Troponin-I	459	20	3	34 (22–39)
Beckman · Access 2 · modified-sensitive cTnI	524	2.5	35	56 (27–100)
OCD · Vitros 3600 · cTnI ES	524	12	2	19 (12–22)
Roche · Cobas e 601 · cTnI	524	160	1	184 (<160–706)
Siemens · Centaur · TnI Ultra	523	6	6	12 (10–16)
Siemens · Dimension EXL 200 · cTnI	524	17	2	34 (17–44)
Siemens · Dimension Vista · cTnI	523	15	1	21 (<15–39)
Siemens · Immulite 2000 XPi · cTnI	479	100	5	392 (190–520)
POC				
Abbott · i-STAT 300 · cTnI	524	20	6	39 (27–53)
Alere · Triage · Cardio3 cTnI	521	10	1	12 (<10–16)
bioMeriéux · Vidas · cTnI Ultra	524	10	1	<10 (<10–40)
IL · GEM Immuno · cTnI	524	1.3	28	15 (10–26)
Siemens · Stratus CS · cTnI	498	30	2	40 (<30–40)

1.4 Biosensors

A biosensor in its simplest sense can be defined as an analytical device in which a biological recognition entity is coupled in close proximity to a transducer (Thevenot et al., 2001). A wide range of biological entities such as enzymes, antibodies and antibody fragments, nucleic acids (DNA, RNA and aptamers), cell receptors, tissues and even whole micro-organisms can be used as the biological recognition element (Tothill, 2009). Similarly a wide range of transducers can be used to convert the chemical change between a receptor and target molecule to a measurable electronic signal output. The range of transducers used include; optical, electrochemical, thermometric, piezoelectric (acoustic) and micromechanical transducers (Tothill, 2009; Holford et al., 2012). As most of the transducers used for CVD detection are focused on optical, piezoelectric and electrochemical detection, these will be reviewed below.

1.4.1 Optical biosensors

Optical biosensors make use of the ability of biological molecules to interact with an electromagnetic field (e.g. light) which travels through the biological molecule, or is reflected from it. The biosensor is designed to measure the change in frequency, amplitude, or polarisation of input light, occurring during the biorecognition process. This response is translated into a quantifiable signal, which is proportional to the amount of the biomolecule present on the sensor surface (Qureshi, 2012).

The largest group of optical biosensors are colorimetric, fluorescence and luminescence biosensors, where the target molecule or biorecognition molecule is labelled with a chromo- or flurophore. These can be very sensitive, able to detect down to nM - fM concentration levels or even a single labelled molecule (Fan et al., 2008; Hasanzadeh et al., 2013). They are also simple to use, are not affected by electromagnetic interference, and can be low cost.

The other big group of optical biosensors are label-free biosensors, such as surface plasmon resonance (SPR) biosensors. SPR biosensors use a "chip" consisting of a prism, coated on one side with a very thin film of metal (most

often gold) to provide a surface to which probe molecules (e.g. antibodies) are bound. They exploit the properties of light totally reflecting internally at a metal-dielectric interface, producing an evanescent field perpendicular to the surface. Although all the light photons are reflected, part of the electromagnetic field penetrates the interface and the energy absorbed from the light photons is transferred to the free electrons in the metal. This causes the formation of surface plasmon polaritons, which are electromagnetic waves (known as electron density waves in quantum theory) propagated in metals through the collective motion of vast numbers of free electrons. These waves travel longitudinally to the dielectric-metal interface, and are known as evanescent waves because the amplitude of the wave decreases exponentially with increasing distance from the interface surface, as shown in Figure 1.9A and B. The effective depth of the evanescent field is about 200 to 300 nm. Since the wave is on the edge of the metal and the external medium interface, these oscillations are very sensitive to any changes in this boundary region, such as the adsorption of molecules to the metal surface.
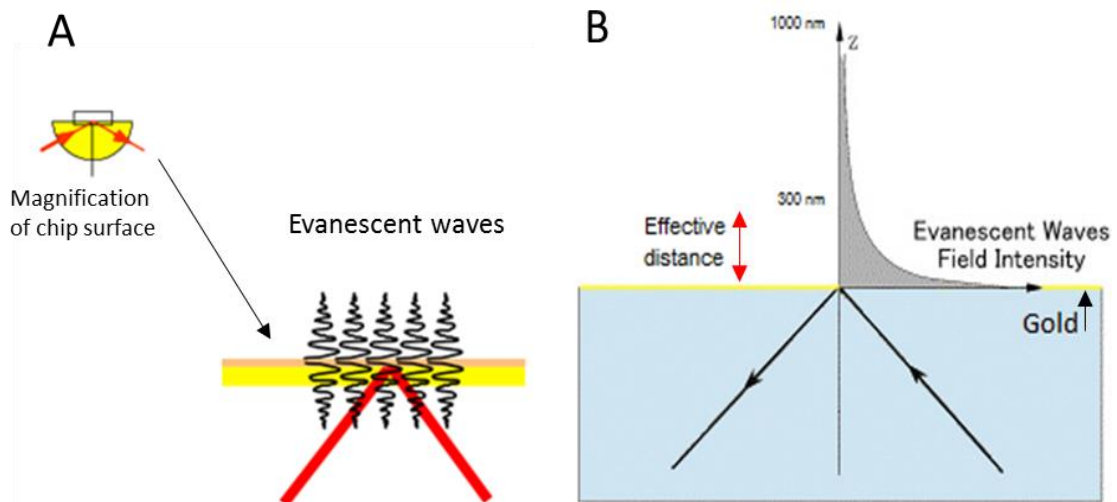
Figure 1.9   Schematic of evanescent waves at dielectric-metal interface

Modified from SPR Theory: Evanescent Wave http://www.sprpages.nl/spr-overview/spr-theory.html and http://www.clinbiomech.com/cms/attachment/2006272301/2028389263/gr1.jpg (accessed 19 Jan 2015)

In a SPR instrument light passes through the prism and reflects off the back side of the sensor surface into the detector which measures its intensity, as the angle of incident light is gradually changed. At a specific angle the momentum and energy of the polarised light will be the same as that of the surface plasmons, exciting them and causing them to resonate (the phenomenon of surface plasma resonance). This angle is called the "resonance angle" (Liedberg et al., 1995). The resulting abrupt loss in reflected light intensity can be seen as a dip in the resonance activity plot Figure 1.10. As the target molecule binds to the antibody, and the protein concentration at the surface increases, the refractive index will change, whilst the refractive index on the prism side will stay constant, leading to a change in resonance angle.
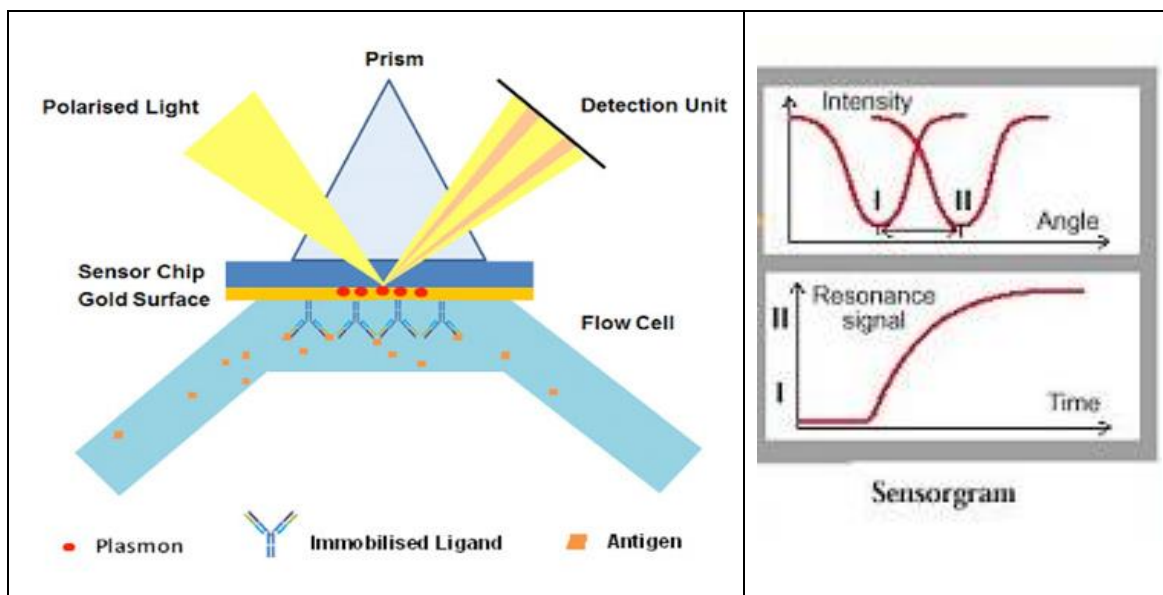
Figure 1.10: Schematic of a Surface Plasmon Resonance Biosensor.

(Reproduced from www.bdi.ie/antibody/spr_biacore.html, accessed 12/12/2013)

The shifts in resonance angle can be exploited to measure the concentration of target molecule in a sample by comparison to reference standards. The great advantage of this technique is that no labelling of molecules is required, it provides real time information, and it can also be used to study binding kinetics measuring affinity and dissociation constants (Schasfoort and Tudos, 2008).

There are many other label-free biosensors being developed using different technologies, which are being applied to the detection of cardiac biomarkers. Some of these are also based on different types of resonance, such as optical fibres, resonant waveguide gratings, resonant mirror interferometry and ring resonators (Fan et al., 2008; Yeom et al., 2011). Another technique being investigated is optomagnetic detection (Dittmer et al., 2010).

1.4.2 Piezoelectric biosensors

Piezoelectric or acoustic biosensors detect the presence of their target analyte by measuring extremely small changes in mass (from *piezo*, the Greek for pressure), down at the pg/mL level. The most well-known type of piezoelectric biosensor is the Quartz Crystal Microbalance (QCM), which utilises a quartz crystal coated with a very thin film of gold. When an electric potential is applied across the crystal, it will resonate at a particular frequency, which is dependent on the cut and thickness of the crystal. Sauerbrey (1959) first showed the relationship between the change in frequency and mass per absolute area was linear and proportional. This idea was developed further and adapted to the use of liquids by Kanazawa and Gordon (1985) by additionally relating viscosity and density of the liquid to the resonance frequency.

A QCM biosensor or immunosensor is created by immobilising a ligand molecule (e.g. antibody) to the gold layer. The change in resonant frequency as the target molecule in solution binds to the ligand is measured, and related to the concentration via a sensorgram (Uludag and Tothill, 2010). Similar to SPR biosensors, QCM biosensors can also be used to study the molecular interactions taking place, determining the association and dissociations constants and affinity.

Another similar biosensor which uses piezoelectric crystals is the Surface Wave Acoustic (SAW) biosensor. Again ligands such as capture antibodies can be applied to the gold surface layer of the crystal. An oscillating voltage is applied to the crystal generating acoustic waves of a specific resonant frequency. The acoustic energy is confined to the surface of the crystal and is very sensitive to perturbations from changes in mass or viscosity, so when the target molecule

interacts there is a change in resonant frequency. This change is measured and output as a signal proportional to the concentration of analyte present (Länge et al., 2008)

1.4.3 Electrochemical biosensors

Clark first started research into biosensors in 1956, which led to the development of the first electrochemical biosensor in 1962, which utilised the enzyme glucose oxidase to measure blood glucose (Clark and Lyons, 1962). The next big development was that of the electrochemical immunosensor (Janata, 1975), which has since found an extremely wide range of applications in the clinical and medical diagnostics, food technology and safety, agriculture, defence and environmental fields, with research and development continuing to expand at an ever increasing rate (Turner, 2013; Sassolas et al., 2012; Salam and Tothill, 2009; Parker and Tothill, 2009).

Immunosensors are based on the binding of an antibody with its antigen, where the complex formed is measured using an electrical signal via the transducer. The inherent affinity and specificity of the antibody-antigen reaction ensures very specific and selective detection of the target molecule. Other advantages of electrochemical immunosensors are; simplicity, robustness, a wide dynamic range, potentially low limits of detection (especially if amplification techniques are used), low cost and they can easily be miniaturised leading to portability. The key disadvantage of immunosensors is that they do not regenerate well. However, this can be overcome by the use of disposable screen printed electrodes (SPE), which are cheap to mass manufacture, so can be disposed of after a single use.

Electrochemical biosensors can be classified into three main types based on the detection transducer used; amperometric, potentiometric and conductimetric (or impedance). In clinical diagnosis, amperometric detection is most commonly used for electrochemical immunosensors (Tothill, 2009). Since biomarkers such as proteins are not electrochemically active, the sandwich ELISA method is employed. A capture antibody is immobilised onto a semiconductor or screen printed electrode, the antigen is captured, then a

second antibody (labelled with an enzyme) binds to the antigen. A substrate is added and the enzyme produces a redox reaction, which produces a measurable current when a fixed potential is applied between the working and reference electrode. Potentiometric detection is more direct, as the potential produced when an analyte binds to immobilised enzyme membrane is measured against a reference electrode, but is rarely used for biomarkers. Recently there has been more interest in impedance detection, as it is a label-free technique that can be used for real time detection, and also studying affinity kinetics (Zheng et al., 2013; Ma et al., 2013). Impedance biosensors measure impedance changes in solution when the target analyte interacts with the biorecognition molecule, which has been immobilised onto an electrode surface (Wang et al., 2012).

1.5 Detection of Troponin and other cardiac biomarkers using biosensors

After over 25 years of ongoing research into alternative cardiac biomarkers, the troponins still prove to be the most important in actual diagnosis of AMI. Many other established and novel cardiac markers have been investigated, but despite numerous clinical trials, they have only been proven to be of value in risk stratification and prognosis of CVD. Thus the development of biosensors for cardiac biomarkers has mainly centred on measuring troponin or troponin plus additional markers which give a potential earlier warning of AMI. All the commercially available devices currently use some form of immunoassay technique, with the choice of detection system dependent on if and how the antibodies are labelled. There have been a number of recent reviews of the use of biosensors to detect cardiac biomarkers (Qureshi et al., 2012, Hasanzadeh et al., 2013, Altintas et al., 2014). The following review concentrates mainly on immunosensors developed for cardiac troponins, especially cTnT which will be the focus of this project. A table summarising the different approaches for detection of cTnT on each sensor platform, their total assay times and limits of detection are summarised in Table 1.3.

Table 1.3: Summary of cTnT detection on different sensor platforms with their total assay times and detection ranges as reported in the literature.

Type of immunosensor	Assay Time	Linear Range	LOD	Reference
Electrochemical				
Antigen – Ab interaction (Capacitance)	360 min	$0.07 – 6.83$ ng mL^{-1}	0.07 ng mL^{-1}	de Vasconcelos et al., 2009
Sandwich immunoassay on Ag SPE (Amperometry)	300 min	$0.10 – 10$ ng mL^{-1}	0.2 ng mL^{-1}	Silva et al., 2010
Carbon nanotubes on gold wires (Potentiometry)	3 days	$1410 – 2086$ ng mL^{-1}	160 ng mL^{-1}	Moreira et al., 2011
Silicon nanowires (Resistance)	1100 min	$0.001 – 10$ ng mL^{-1}	0.001 ng mL^{-1}	Zhang et al., 2011
Carbon nanotubes on polyethyleneimine (Amperometry)	300 min	$0.10 – 10$ ng mL^{-1}	0.1 ng mL^{-1}	Gomes-Filho et al., 2013
An *o*-aminobenzoic acid film-based immunoelectrode (Amperometry)	380 min	$0.05 – 5.0$ ng mL^{-1}	0.016 ng mL^{-1}	Mattos et al., 2013
Sandwich assay using magnetic beads (Amperometry)	120 min	$0.05 – 1$ ng mL^{-1}	0.017 ng mL^{-1}	de Avila et al., 2013
o-phenyldiamine MIP (Potentiometry)	Not supplied	$0.009 – 0.8$ ng mL^{-1}	0.009 ng mL^{-1}	Karimian et al, 2013
Carbon nanotubes on SPE (Amperometry)	205 min	$0.0025 – 0.5$ ng mL^{-1}	0.0035 ng mL^{-1}	Silva et al., 2013
Chitosan stabilised AuNPs (Voltammetry)	≥100 min	$0.20 – 100$ ng mL^{-1}	0.1 ng mL^{-1}	Brondani et al., 2014
AuNP enhanced SPE (Amperometry)	120 min	$0.39 – 100$ ng mL^{-1}	0.58 ng mL^{-1}	Fakanya, 2012
Optical				
Cysteamine-glutaraldehyde SAM, SPR, label free	112 min	$0.05 – 4.5$ ng mL^{-1}	0.05 ng mL^{-1}	Dutra et al., 2007b
Streptavidin SAM & Biotin-labelled anti-cTnT-Mab, SPR, label-free	>45 min	$0.03 – 6.5$ ng mL^{-1}	0.01 ng mL^{-1}	Dutra et al., 2007a
Dextran sensor chip, SPR, sandwich assay	>46 min	$10 – 100$ ng mL^{-1}	10 ng mL^{-1}	Andersson et al., 2010
OEG mixed SAM, SPR, label free	Approx. 900 min	$100 – 50000$ ng mL^{-1}	100 ng mL^{-1}	Liu et al., 2011
Piezoelectric				
QCM using PVC sensor coating	Not supplied	Not supplied	5 ng mL^{-1}	Wong-ek et al., 2010
QCM using immobilised AuNPs	1400 min	$0.003 – 0.5$ ng mL^{-1}	0.0015ng mL^{-1}	Fonesca et al., 2011
Dual – QCM, label-free	196 min	$0.025 – 4.0$ ng mL^{-1}	0.008 ng mL^{-1}	Mattos, et al., 2012

1.5.1 Electrochemical biosensors

In healthy individuals baseline troponin levels (taken as 99th percentile) are approximately <60 pg mL^{-1} (Apple et al., 2012), <30pg mL^{-1} (Babuin and Jaffe, 2005). This level of sensitivity is very difficult to achieve using simple amperometric electrochemical biosensors, such as screen printed electrode (SPE) immunosensors. For cardiac biomarkers which have inherently much higher baseline levels, such as CRP (0.1 - 0.8 mg mL^{-1}) and H-FABP (4.8 to 9.1 ng ml^{-1}), the development of suitable electrochemical biosensors is more realistic and achievable (Casa et al., 2008; Viswanathan et al., 2012). Therefore, different, ever more complicated modifications to electrochemical based immunosensors have been investigated to try to achieve the sensitivity required for clinically diagnostic troponin measurement.

Silva et al (2010) used a SPE immunosensor with a streptavidin/avidin system to amplify the signal, and managed to obtain a limit of detection (LOD) of 200 pg mL^{-1} cTnT. The use of self-assembled monolayers and gold nanoparticles (AuNPs) has been well documented in improving the sensitivity of (electrochemical) immunosensors (Siangproh et al., 2011; Saha et al., 2012, Ding et al., 2013). This amplification system was investigated by Fakanya (2012) in the development of a SPE amperometric immunosensor for cTnT, but the best LOD achieved was only 510 pg mL^{-1} in buffer and 580 pg mL^{-1} in a 75% serum solution. Another approach to signal amplification using carbon nanotubes (CNT) on SPEs, was taken by Gomes-Filho et al., (2013), which obtained a LOD of 100 pg mL^{-1}. However, they did have to label the detection antibodies with peroxidase (to get an amperometric signal). The use of CNT-SPE was developed further by using CNT functionalised with amine groups (Silva et al., 2013). This enabled better control of the density and orientation of the CNTs on the sensor surface, and a stronger covalent immobilisation of the antibodies via the amide bond. Using differential pulse voltammetry, the group was able to obtain a LOD of 7 pg mL^{-1} cTnT in serum samples (Silva et al., 2013).

The usefulness of molecular imprinting technology has also been examined in the development of immunosensors for cTnT. Moreira et al., (2011) imprinted a cTnT template onto multi-walled carbon nanotubes. This material was then mixed with plasticiser and coated onto silver, gold and titanium wires. Using a gold wire electrode they managed to measure cTnT with great specificity, but rather poor sensitivity at 160 ng mL^{-1}. Recently Karimian et al., (2013) used a different angle, and also investigated a molecularly-imprinted biosensor for cTnT measurement. The molecular imprinting was carried out using electropolymerisation. The binding of cTnT was then measured using cyclic voltammetry. A linear response was obtained, permitting sensitive detection of cTnT down to 9 pg mL^{-1}. Brondani et al., (2014) also used voltammetry for their immunosensor, but it was a very different approach, as chitosan was used to stabilise AuNPs, which were then bound to anti-cTnT capture antibodies. This chitosan-AuNP-anti-cTnT complex was then bound to a novel ionic organic film (I-Py) which had been coated onto the electrode. This enabled detection of cTnT with an LOD of 100 pg mL^{-1}.

A simpler amperometric immunosensor has been published by Mattos et al., (2013), which involved the application of a stable film of *o*-aminobenzoic acid to the sensor surface using electropolymerisation. The capture antibody was then attached covalently to this film. They found this electrode to be very stable and conductive, allowing them to measure cTnT down to an LOD of 16 pg mL^{-1}.

Other electrochemical detection techniques have also been studied. A label-free capacitance based immunosensor was developed by de Vasconcelos et al., (2009), which used oxidised silicon wafers coated with capture antibodies as the electrodes, with a capacitance meter. This allowed detection of cTnT down to 70 pg mL^{-1} in human serum, and in theory it should be relatively easy to miniaturise the device and make is portable. Since then, Bhalla et al., (2012) utilised capacitance detection with citrate capped AuNPs to measure cTnI, which enabled label free detection down to a LOD of 200 pg mL^{-1}. Detection using resistance change was the method employed by Zhang et al., (2011), to measure cTnT down to 1 pg mL^{-1} using a silicon nanowire array biosensor chip,

with a sample volume of 2 μL of blood. Periyakaruppan et al., (2013) employed a resistance method (electrochemical impedance spectroscopy) using vertically aligned carbon nanofibres, to measure cTnI with a sensitivity of 200 pg mL^{-1}.

As new analytical technologies are developed, there is always a great interest in how these may be adapted to the measurement of cardiac troponins. The use of magnetic beads was recently investigated by de Avila et al., (2013) to develop a disposable immunosensor. They bound biotinylated cTnT capture antibodies to streptavidin coated magnetic beads, and used a horse-radish peroxidase labelled detection antibody. The magnetic beads are then magnetically captured on a gold SPE. Commercial TMB/H_2O_2 reagent solution is used to mediate the enzyme reaction on the electrode, and the resulting current is measured amperometrically. Using this system de Avila et al., were able to detect down to 17 pg mL^{-1} cTnT.

Another novel approach was taken by Zhou et al., (2010), using a film of polydimethylsiloxane-AuNPs and antibodies bound to quantum dots to enhance the sensitivity, and employing anodic stripping voltammetry for detection. The LOD achieved was 10 pg mL^{-1} for both cTnI and CRP. Yet another methodology enabled the most sensitive detection of cTnI to date, down to 100 fg mL^{-1} (without) and 10 fg mL^{-1} (with) redox cycling. Akanda et al., adsorbed avidin onto indium-tin oxide electrodes and used phosphatase conjugated antibodies, both with and without a reducing agent tris(2-carboxyethyl)phosphine (Akanda et al., 2011).

1.5.2 SPR Immunosensors

The use of SPR for the development of troponin immunosensors was first examined in 2007, although the previous year the technique had been investigated for analysis of the cardiac biomarkers CRP and BNP (Casa et al., 2006; Kurita et al., 2006). Both groups used a self-assembled monolayer (SAM) to functionalise the surface of the chip, and then covalently immobilise the antibodies. The method of Masson et al., (2007) quantified two cardiac biomarkers, myoglobin and cTnI using a SAM of N-hydroxysuccinimide activated 16-mercaptohexadecanoic acid. This helped to reduce non-specific

binding of serum proteins, and enabled detection of these markers in undiluted serum down to 700 pg ml^{-1} and 900 pg mL^{-1} for cTnI and myoglobin, respectively. Dutra et al., (2007 a,b) used a cysteamine and glutaraldehyde system to prepare the SAM and couple it to the anti-cTnT antibody, and also regenerated the sensor surface by applying a dilute SDS solution. This enabled detection of cTnT down to 50 pg mL^{-1} with good reproducibility.

Other SPR approaches include that by Lui et al., (2011), which used a mixture of oligoethyleneglycol-terminated alkanethiolate and mercapto-hexadecanoic acid for the SAM. Although this reduced non-specific protein binding, it also reduced the sensitivity obtained to only 100 ng mL^{-1}, making it unsuitable for use in clinical diagnosis. Rather than use a standard anti-cTnI antibody, Kwon et al., (2011) prepared an epitope peptide against a specific region of cTnI (aa 84-94). This peptide was cross-linked onto the sensor surface, and the SPR signal analysed, however, this set up only managed to obtain an LOD of 68 ng mL^{-1}. A more successful approach was taken Andersson et al., (2010), who used a commercial carboxymethyl hydrogel coated sensor chip instead of applying a SAM, and developed a sandwich assay based immunosensor for studying cardiotoxicity. Using this technique they were able to get limit of detection of 10 ng ml^{-1}, which was sufficient for assessing cardiotoxicity.

1.5.3 QCM and SAW immunosensors

In recent years there have been three reports of using QCM for the detection of troponins. Wong-ek et al., (2010) developed a QCM immunosensor using a carboxylic polyvinyl chloride coating on the sensor surface, onto which antibodies were immobilised. This enabled detection of cTnT down to 5 ng mL^{-1}. A significantly more sensitive cTnT detection method using AuNPs for amplification was published the following year by Fonesca et al., (2011). The AuNPs were immobilised onto the electrode sensor surface using thiol-aldehyde linkages, to which anti-cTnT antibodies were also co-immobilised. The detection limit in serum samples using this system was 3 pg mL^{-1}. The same group later developed a dual QCM system, consisting of two quartz crystal immunosensors. One quartz crystal is used as a working electrode, and

the other which has no anti-cTnT attached, is used as the reference electrode. Using a reference electrode has the advantage that any non-specific binding is compensated for. This dual QCM system allowed accurate detection of cTnT down to 8 pg ml^{-1} in undiluted serum samples, and the results correlated well with those obtained from a conventional immunoassay method (Mattos et al., 2012).

Immunosensors based on using SAW detection have also been utilised for the analysis of troponins. Initially a multi cardiac marker SAW immunosensor was developed for myoglobin, CK-MB and cTnI, using AuNPs conjugated to detection antibodies, which were immobilised on the sensor surface. The AuNPs and gold staining system used, helped to amplify the signal, enabling detection down to 16 ng mL^{-1}, 1.1 ng mL^{-1} and 20 pg mL^{-1} for myoglobin, CK-MB and cTnI, respectively (Lee et al., 2011). This group have since further refined their method and instrumentation concentrating on cTnI only, to develop a fully automated POCT device, which uses centrifugation and a disposable microfluidic cartridge. This POCT device allows rapid, reproducible, accurate and sensitive measurement of cTnI down to 10 pg mL^{-1} (Lee et al., 2013 a, b).

1.6 Aim and Objectives

Global statistics show that cardiovascular disease is already the world's biggest cause of mortality. The earlier diagnosis of AMI and myocardial damage will help to reduce mortality and morbidity, allowing the correct and most suitable treatment to be initiated as soon as possible. This reduces healthcare costs and the economic burden from CVD to society, focussing limited resources where needed, as well as improving the quality of life for the people affected.

To achieve this there is increasingly a demand for the diagnosis to be more localised, leading to a trend for smaller, simpler instruments which are portable and easy to use. At the same time technology has advanced, allowing biosensors to be developed, which are faster, more accurate, and amenable to miniaturisation, so that small, portable devices can be built.

The biomarker chosen for this project was cTnT for two reasons. Due to the early patenting of the antibody by Roche, the epitopes for cTnT have been standardised and are used industry-wide, thus all assays for cTnT are comparable. The cTnT is present as a single molecule, not different combinations of complexes as is cTnI. This allows the use of a single capture and a single detector antibody, making any future immunosensor simpler and more cost effective to produce (as for accurate cTnI detection you need four antibodies of different epitopes to bind to all the different combinations of complexes).

Therefore, the aim of this project is to develop a sensitive immunosensor for the determination of the cardiac biomarker Troponin T concentrations in human serum, for use in the early diagnosis of cardiovascular disease, especially AMI, which could be further developed into a point-of-care test.

Initially an immunoassay will be developed for the analysis of cardiac troponin T in buffer using optical detection with an SPR instrument. The immobilisation of capture antibodies will be optimised, as well as other assay conditions, to maximise the sensitivity of the immunosensor. Further signal enhancement will be explored through the development of a sandwich format, and also the amplification of the signal through the use of gold nanoparticles. The use of the immunosensor in serum will be optimised, and the performance of the final immunosensor will be evaluated in human serum. A flow chart of the different developmental phases is presented in Figure 1.11.

Chapter 1

Basic Introduction and Literature review

Chapter 2

Development of an assay for Cardiac Troponin T (cTnT) using SPR for optical detection

Optimisation of SAM
Optimisation of immobilisation conditions
Optimisation of assay conditions
Comparison of Direct and Indirect assays for cTnT in buffer

Chapter 3

Optimisation of assay for determination of cTnT in human serum

Selection of control antibody
Investigation of non-specific binding and use of additives to minimise it
Investigation of non-specific binding in human serum
Re-optimisation of detector antibody
Use and optimisation of gold nanoparticles for signal amplification
Assessment of final assay method in human serum

Chapter 4

Final conclusion and Future Work

Figure 1.11:   Flow chart outlining the different phases of the work carried out in this study.

CHAPTER 2

DEVELOPMENT OF AN SPR ASSAY FOR CARDIAC

TROPONIN T

2 DEVELOPMENT OF AN SPR ASSAY FOR CARDIAC TROPONIN T

2.1 Introduction

This chapter describes the investigations performed to develop and optimise an SPR-based immunoassay to detect cardiac Troponin T (cTnT) protein using a Sierra Sensors SPR-4 sensor system.

The surface chemistry applications used to functionalise biosensor chips play a critical role in obtaining a good detection signal from the system. If anti-cTnT antibodies are directly adsorbed onto the gold surface of the sensor, they may become denaturised, or their orientation may prevent the binding of sufficient cTnT molecules, by blocking the active binding sites. The use of physical adsorption would also give a weak interaction, and reduce the reuse of the sensor, due to the less stable surface resulting in the loss of antibodies.

Therefore, capture antibody is attached to the sensor surface using covalent interactions which avoids these problems. This is achieved by forming a self-assembled monolayer (SAM) on the gold surface of the sensor chip, using a molecule which has an exposed active group on the end. One of the most common classes of molecules used is the alkanethiols, which have a spontaneous strong interaction between their sulphur group and the gold surface (Love et al., 2005). The alkanethiols interact with the gold surface forming a monolayer. The monolayer is further stabilised and ordered by the van der Waals forces between the long hydrocarbon chains.

For the development of this assay, 11-mercaptoundecanoic acid (11-MUDA) was used, which has 11 carbon atoms and provides a carboxylic acid group as the active group when it forms the SAM (Figure 2.1). The appropriate length of alkanethiol molecules changes depending on the sensor type. For example, short chain lengths perform better for electrochemical sensors, whereas longer chain lengths are more appropriate for SPR and QCM-based systems.
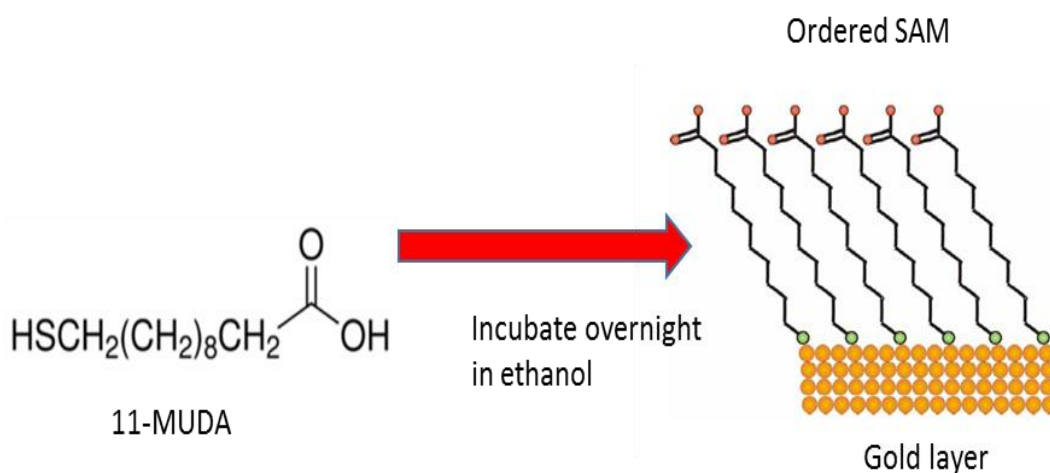
Figure 2.1: The structure of 11-MUDA (11-mercaptoundecanoic acid), with a schematic of how it forms a SAM on the sensor's gold surface.

After SAM formation, the sensor surface was activated using conventional amine coupling chemistry to immobilise the antibody covalently (Salam and Tothill, 2009). For this, the carboxylic acid groups of the 11-MUDA forming the SAM on the sensor surface are activated using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) to form amides or esters, as shown in Figure 2.2. The EDC reacts with the carboxyl groups (Step 1). This can be performed in organic or aqueous solutions, as EDC is a water soluble carbodiimide, however water was used to ensure compatibility with the rest of the antibody immobilisation procedure. The N-hydroxysuccinimide (NHS), mediates the formation of an amine-reactive NHS ester (Step 2), which then reacts with primary amine groups on the antibody forming a covalent bond (Step 3). The NHS helps to stabilise the active carbodiimide intermediate in aqueous solutions, controlling the cross-linking reaction and improving its efficiency.

44
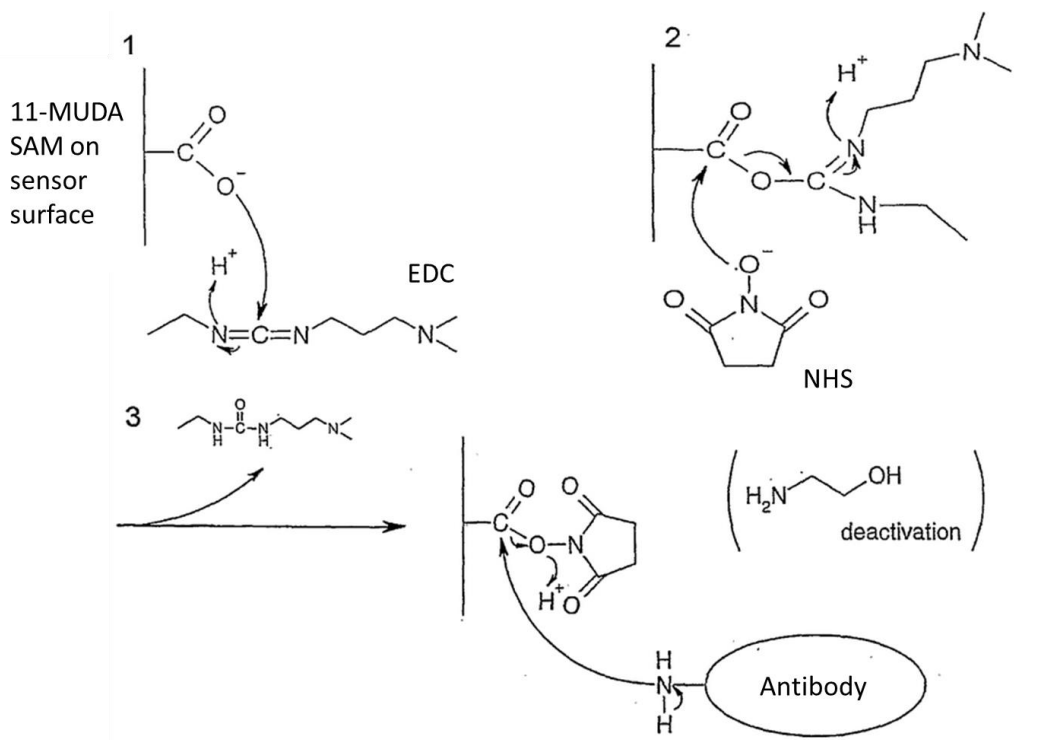
Figure 2.2: Schematic to show activation of sensor surface via carbodiimide coupling chemistry and the formation of a covalent bond with the antibody.

Figure is a modified version taken from Patent No. WO2006088425 A1 (Olofsson et al., 2006).

Two different immunoassay formats (direct assay and sandwich assay) were then examined to evaluate their performance for developing a highly sensitive SPR immunosensor for cTnT detection. A direct assay is where the antigen binds directly to the sensor surface, or more commonly, a capture antibody is immobilised on the sensor surface, and the antigen (analyte) binds to the capture antibody, resulting in a signal increase which can be correlated to the concentration of the analyte in the sample (Figure 2.3). The capture antibody should always be very specific and have a high affinity for the antigen.

In a sandwich assay format, capture antibodies are again immobilised on the sensor surface. A sample is incubated with, or passed over the sensor surface, during which the analyte binds to the capture antibody, as previously with the direct assay. At its simplest a sandwich assay involves a second step where a second antibody is applied, which binds to the analyte, or the antigen-antibody

complex. Thus the analyte is "sandwiched" between the two antibodies, and any excess second antibody is washed away (Figure 2.3). For ELISAs, the second antibody or "detection" antibody would be labelled in some way such as with an enzyme or fluorophore. However, when SPR optical detection is used, there is no need for labelling, as when the detection antibody binds to the immobilised antigen-antibody complex, the resonance angle is altered by a greater amount. This results in measurable signal amplification, which is proportional to the amount of analyte present.
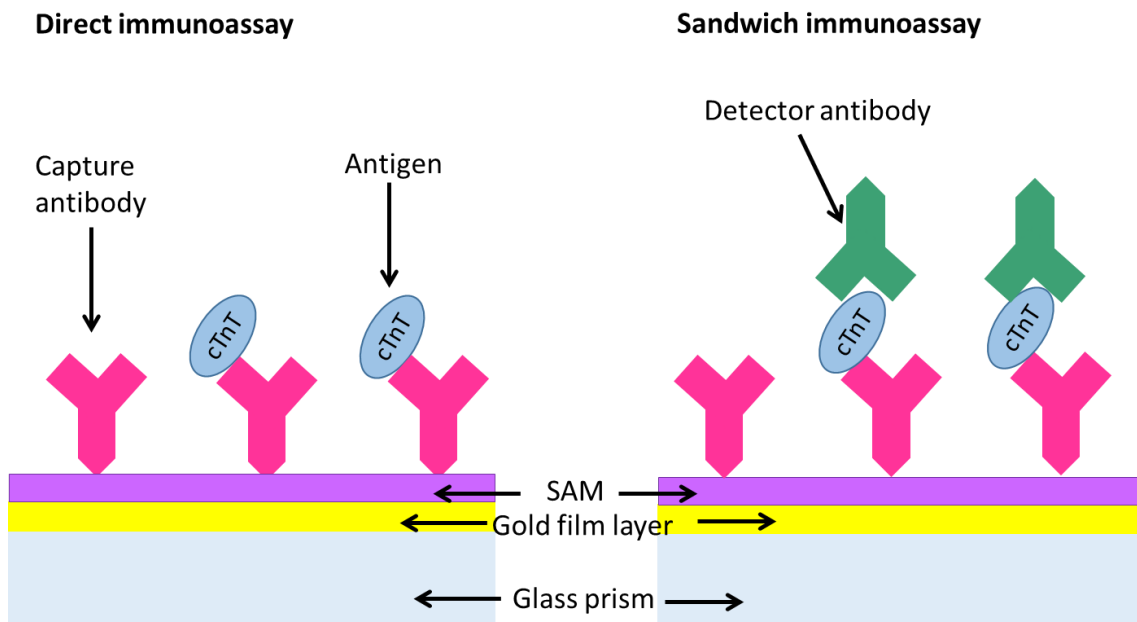
Figure 2.3: Schematics of Direct and Sandwich Immunoassays on an SPR sensor chip.

2.2 Materials and Equipment

2.2.1 Materials

Phosphate buffered saline (PBS, 0.01M phosphate buffer and 0.137M sodium chloride, pH 7.4) tablets, Tween 20, bovine serum albumin (BSA), sulphuric acid, ethanolamine, hydrochloric acid (HCl), hydrogen peroxide, sodium

hydroxide (NaOH), N-hydroxysuccinimide (NHS), ethanol and 11-mercaptoundecanoic acid were purchased from Sigma-Aldrich (Poole, UK). 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) was purchased from Pierce-Thermo (Cramblington, UK). Osmosis (RO, Ultrapure) water (18 M MΩcm-1) was obtained from a Milli-Q water system (Millipore Corp., Tokyo, Japan). The rest of the reagents were of analytical grade.

Anti-Cardiac Troponin T Capture Antibody (Mouse monoclonal (1C11) to cardiac Troponin T : ab8295), Anti-Cardiac Troponin T Detection Antibody (Mouse Monoclonal (7G7) to cardiac Troponin T : ab10223) and Cardiac Troponin T full length protein : ab9937 were all purchased from Abcam (Cambridge, UK).

2.2.2 Equipment

Micropipettes were purchased from Eppendorf (Loughborough, UK). Pipette tips and 1.5 mL tubes were purchased from Fisher Scientific (Loughborough, UK). Glass ware and volumetric materials (flasks, vessels etc.) were of analytical grade. Whatman Filter paper No 1, 11 µm, was purchased from GE Healthcare Life Sciences (Little Chalfont, UK).

A fully automated SPR-4 biosensor, amine coated and high density amine (dextran based) chips from Sierra Sensors GmbH (Hamburg, Germany), were used to develop an immunosensor. Although the sensor chips are referred to as "amine" by the manufacturer, the functional group at the uppermost surface of the SAM layer is actually a carboxyl group. The manufacturer has named them "amine", as they are used to bind to amine groups on target proteins. This sensor system has four sensing spots which allow the performance of four separate assays simultaneously. All data produced by the SPR-4 were further analysed with Microsoft Excel.

2.3 Methods

2.3.1 Cleaning of sensor chip surface

Prior to depositing a SAM on a used commercial chip, the surface of the gold film on the sensor chip was cleaned (to remove any bound material and remaining previous SAM) using piranha solution. The sensor chip was washed with deionised water, followed by ethanol and then dried thoroughly under a stream of nitrogen. The piranha solution was prepared by carefully adding 4 mL of 35% hydrogen peroxide to 12 mL of concentrated sulphuric acid, whilst following all the appropriate safety procedures. The piranha solution was then applied onto the gold surface of the sensor using a glass pipette, until the entire sensor surface was covered, and the solution was left on for 20 minutes. After this incubation step the sensor chip was washed at least three times using deionised water, prior to a final rinse with ethanol. The chip was dried under a stream of nitrogen and then stored at 4°C, until required. Both storage "as is", and in ethanol were tested.

2.3.2 Optimisation of the buffer pH used for immobilisation

Each affinity ligand has an optimal pH for the best performance during binding assays. This pH plays a crucial role on both immobilisation and analyte detection. Therefore, anti-cTnT 1C11 antibody solutions were prepared using 10 mM sodium acetate buffer at different pH (pH 4.5, 5.0, 5.5 and 6.0), at a nominal concentration of 50 µg mL^{-1}. During these pre-concentration tests, the sensor surface was not activated by EDC-NHS chemistry and passive adsorption of the capture antibody on the non-activated sensor chip was used. These solutions in different pH buffers were injected in turn across the surface of the sensor chip for 3 minutes and the response (RU) changes were recorded.

2.3.3 Optimisation of the capture antibody concentration

After determination of the best pH condition for anti-cTnT 1C11 antibody, the best concentration for antibody immobilisation was investigated. Anti-cTnT 1C11 antibody solutions were prepared using sodium acetate (10 mM, pH 4.5) buffer at concentrations of 25, 50 and 75 µg mL^{-1}. The experiment was later

repeated in triplicate by using three different channels on a new sensor chip at concentrations of 10, 20, 40, 60 and 80 µg mL^{-1} of anti-cTnT 1C11 antibody. The different concentrations were then injected for 3 minutes across the sensor surface at a flow rate of 25 µLmin^{-1}, with the capture antibody being passively adsorbed onto the surface. The resulting changes in response (RU) were recorded for each injection.

2.3.4 Immobilisation of antibody to sensor surface

The SPR sensor chip was first docked into the SPR-4 instrument, and primed with PBS running buffer (10 mM phosphate buffer, 0.0027 M potassium chloride, 0.137 M sodium chloride), at a flow rate of 25 µLmin^{-1}. A flow rate of 25 µLmin^{-1} was used throughout this series of experiments. The sensor chip surface was activated with a 1:1 mixture of 0.4 M EDC and 0.1 M NHS. Both reagents had been previously prepared with double distilled deionized water and stored at -20°C. They were mixed immediately prior to use. The EDC:NHS mixture was injected across the surface of sensor spot 1, for 3 minutes. Next, a 50 µg mL^{-1} anti-cTnT (1C11) antibody solution (in 10mM, pH 4.5 sodium acetate buffer) was injected for 3 minutes to activate the surface. Following immobilization of the capture antibody, the sensor surface was blocked by injecting 50 µg mL^{-1} BSA (in PBS buffer) solution for 3 minutes. Non-reacted NHS esters were then capped by injecting 1M ethanolamine solution (pH 8.5) for 3 minutes. The response (RU) changes were recorded two minutes after the protein injection was completed.

2.3.5 Deposition and optimisation of the SAM

The cleaned sensor chip was placed in a clean petri dish for SAM coating. The appropriate concentration of 11-MUDA solution was prepared in ethanol. Three different concentrations of 11-MUDA were investigated; 2, 4 and 10 mM.

The 11-MUDA solution was then added to the petri dish, until the surface of the sensor chip was fully covered. The dish was covered with parafilm and a layer of aluminium foil (to protect it from light) and then incubated at room temperature overnight. The following morning the sensor chip is rinsed with

ethanol and deionised water, then dried under stream of nitrogen. The coated chip was either used immediately, or stored at 4°C in a container sealed with parafilm and protected from light until required. To test the performance of each 11-MUDA concentration, the prepared chip was immobilised with 50 µg mL^{-1} capture antibody (anti-cTnT 1C11 antibody), then one of the three fixed concentration cTnT solutions were injected onto the sensor surface, and the SPR binding response was measured.

2.3.6 Preliminary binding test for cTnT

A 1.55 mg mL^{-1} cardiac Troponin T (full length protein) stock solution was diluted with PBS buffer to prepare the concentrations of 250, 500 and 1000 ng mL^{-1}. The cTnT samples were injected onto a SPR sensor chip which had previously been immobilised with 50 µg mL^{-1} capture antibody (anti-cTnT 1C11 antibody).

The analyte test solution was injected into the SPR for 3 minutes for association, and then allowed to dissociate for 30 seconds. To regenerate the sensor surface prior to the next injection of test analyte, 0.1M HCl and then 20 mM NaOH solutions were injected onto the surface for 1 minute (with a dissociation time of 1 second). Test analyte solutions were injected in ascending order of concentration. During these preliminary tests, PBS was used as the running buffer for the SPR system.

2.3.7 Optimisation of SPR running buffer

To evaluate the most suitable running buffer, the preliminary binding test was first carried out using PBS. The preliminary binding test was then repeated using PBS with 0.05% Tween 20 added (PBS/T), to assess the effect of detergent on binding. The microfluidics of the sensor system was initially primed using PBS/T to remove PBS buffer from the system and the analyte testing was then started. The two sets of results were then compared.

2.3.8 Direct Immunoassay for detection of cTnT

A set of cTnT samples were prepared across the concentration (25 – 1000 ng mL^{-1}). The samples were simultaneously injected across across all four spots in ascending order of concentration. The surface was regenerated between sample injections by injecting 0.1M HCl (1 minute) followed by 20 mM NaOH (1 minute). The whole experiment was repeated three times, over a period of 30 hours, to evaluate the stability of the sensor conditions with time.

2.3.9 Sandwich Immunoassay for detection of cTnT

A sandwich immunoassay format was investigated to improve the limit of detection (LOD) using signal amplification via the binding of a detection antibody to the bound cTnT. A test solution of cTnT was prepared at a concentration of 200 ng mL^{-1}. The test cTnT solution was injected for 3 minutes. After each injection of solution, a solution of detection (secondary) anti-cTnT (7G7) antibody at a fixed concentration of 20 µg mL^{-1}, was injected for 3 minutes. The sensor was then regenerated by injections of 0.1M HCl and 20 mM NaOH after each injection of detection antibody solution.

2.3.10 Optimisation of detection antibody concentration

The optimal concentration of the detection antibody was investigated by preparing a set of different anti-cTnT (7G7) antibody concentrations; 1, 2, 4, 8 and 16 µg mL^{-1}. The samples were prepared from the 20 µg mL^{-1} stock anti-cTnT (7G7) antibody solution, using running buffer (PBS/T).

During these optimisation tests, a fixed concentration of cTnT (100 ng mL^{-1}) was used. This was injected for 3 minutes prior to the injection of the detection antibody solution at the given concentrations. After each injection of detection antibody, the sensor chip was regenerated using 0.1M HCl and 20 mM NaOH, prior to the next injection of cTnT followed by the next detection antibody concentration until the full set of concentrations were completed.

2.3.11 Calculation method

Coefficients of variation (CVs) were calculated using the equation below, as the ratio of standard deviation (σ) to the mean (μ).

$$\% \, CV = \frac{\sigma}{\mu} \; x \; 100$$

The lower the %CV, the less dispersion there is in the variable, and thus the more precise the assay.

The limit of detection was calculated as the response obtained from the cTnT concentration that is equivalent to 3 times the standard deviation of the responses obtained from the blank standards (which were injections of the sample preparation buffer only).

2.4 Results and Discussion

2.4.1 Optimisation of the buffer pH used for immobilisation

The pH at which the immobilisation of the capture antibody is carried out is important for two reasons. Firstly, the pH affects the reaction rate of the EDC/NHS reaction, which only works between pH 3.5 and pH 8.5. Secondly, the correct pH allows the pre-concentration process to take place. The pre-concentration process is where the ligand is uncharged, letting it move close to the sensor surface, which then enables the amino groups on the ligand to react with the activated ester group.

The optimum pH of the sodium acetate buffer used to prepare immobilisation antibody was investigated using passive adsorption by examining the SPR response obtained when capture antibody was injected onto the sensor chip surface in the SPR, whilst varying the pH of the immobilisation buffer. The capture antibody solution was prepared at a fixed concentration of 50 μg mL^{-1} using 10 mM sodium acetate at different pHs (4.5, 5.0, 5.5 and 6.0).

Table 2.1: Effect of immobilisation buffer pH on binding responses of anti-cTnT 1C11 antibody (capture antibody).

pH of 10 mM sodium acetate buffer	SPR Response (RU)	
	1st injection	2nd injection
4.5	56.1	49.1
5.0	49.1	42.5
5.5	40.5	-
6.0	19.3	-

The response was found to decrease as the pH was increased, as shown in Table 2.1. The optimum pH was found to be pH 4.5, and this was used for all future immobilisation of capture antibody. Although potentially, the response may have been increased even more by lowering the pH further, it was decided not to pursue this, as decreasing the pH further may start to affect the stability of the immobilized antibody by beginning to denature it.

An optimum pH of 4.5, is supported by the fact that to get positive charges on the amino group, the pH of the solution needs to be 0.5 to 1 pH unit below the isoelectric point of the molecule (Fischer, 2010). However to maintain the negative charge of the carboxyl (activated ester) group on the sensor surface, the pH needs to be greater than pH 4. The isoelectric point of cTnT is 4.98 (Signaling Gateway database, 2014), so the best compromise pH will be pH 4.5, which correlates well with the obtained experimental results.

2.4.2 Optimisation of the capture antibody concentration

The optimum concentration of capture antibody to be immobilised was evaluated using passive adsorption and the previously optimised immobilisation buffer (10 mM, pH 4.5 sodium acetate). A pre-coated standard amine sensor chip from Sierra Sensors was used for both experiments. The concentrations tested were 25, 50 and 75 µg mL^{-1} of anti-cTnT 1C11 antibody for the first optimisation experiment and 10, 20, 40, 60 and 80 µg mL^{-1} for the second optimisation test. The experiment was repeated with more data points and replicates to confirm the optimum concentration chosen was appropriate.

The binding response increases linearly against antibody concentration from to 20 μg mL^{-1} up to the 50 – 60 μg mL^{-1} concentration level, after this the increase in response per extra unit of concentration is decreased, as shown in Figure 2.4. A 50 μg mL^{-1} concentration of anti-cTnT (1C11) antibody was chosen as the optimum concentration for the capture antibody, as this was the best compromise between a high response and cost effective use of the antibody.
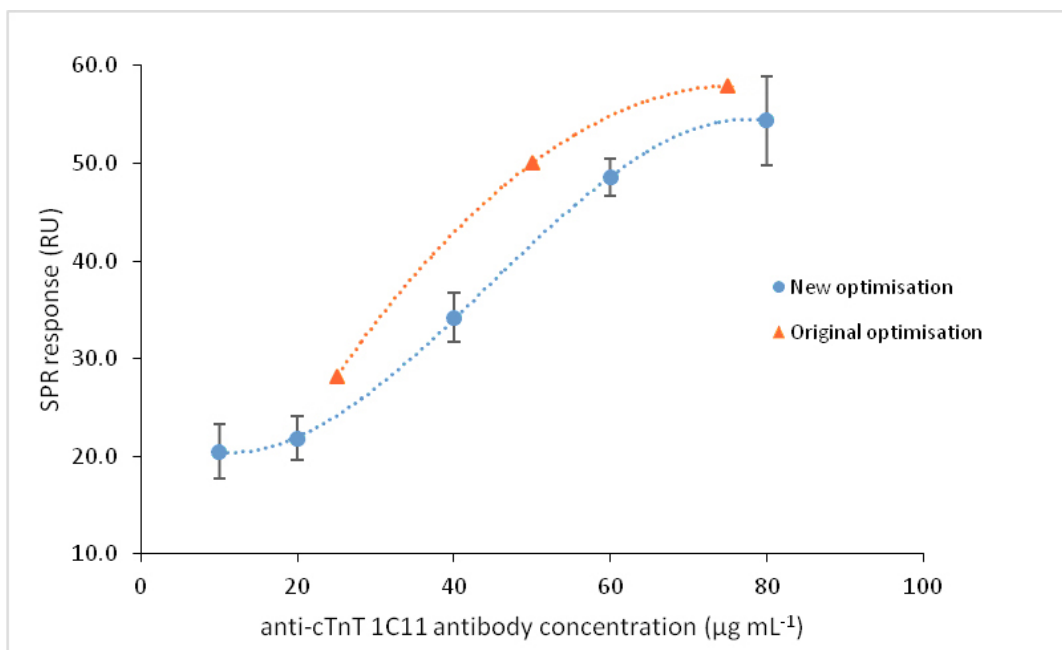
Figure 2.4: Effect of concentration of anti-cTnT (1C11) antibody immobilised on SPR response. 10 - 100 μg mL^{-1} anti-cTnT (1C11) antibody solution (sodium acetate 10 mM, pH 4.5) immobilised for 3 minutes at 25 μL min^{-1} using passive adsorption. Error bars represent SD (n=3).

2.4.3 Immobilisation of antibody on the sensor surface

Once the concentration and pH for the immobilisation procedure were optimised, the optimal conditions were used to immobilise the capture antibody onto sensor surface covalently. The sensor surface was flushed with running buffer, then injected with a freshly prepared mixture of EDC and NHS, to activate the surface. The sensorgram in Figure 2.5 shows the typical binding response profile obtained on activation of the sensor surface, followed by immobilisation of the antibody, then blocking with BSA and ethanolamine. There is an initial bulk shift when the EDC/NHS is injected, which is due to the

difference in refractive index between the concentrated EDC/NHS mixture and the running buffer. Any unbound EDC/NHS is washed off by the flow of running buffer. The injection of antibody then gives a positive shaped binding curve (as the antibody binds to the active NHS ester groups), followed by a dissociation curve as unbound antibody is washed off. The shift which occurs when changing to running buffer is due to a change in refractive index (when immobilisation buffer (i.e. 10 mM sodium acetate pH 4.5) changes to the PBS/T running buffer. BSA was injected to bind to any unoccupied sites, but there was negligible binding, suggesting that the sensor surface has been effectively coated with capture antibody. There is another high bulk shift during the injection of the 1M ethanolamine, which again was due to the refractive index change caused by the high ionic strength and pH of the ethanolamine solution.
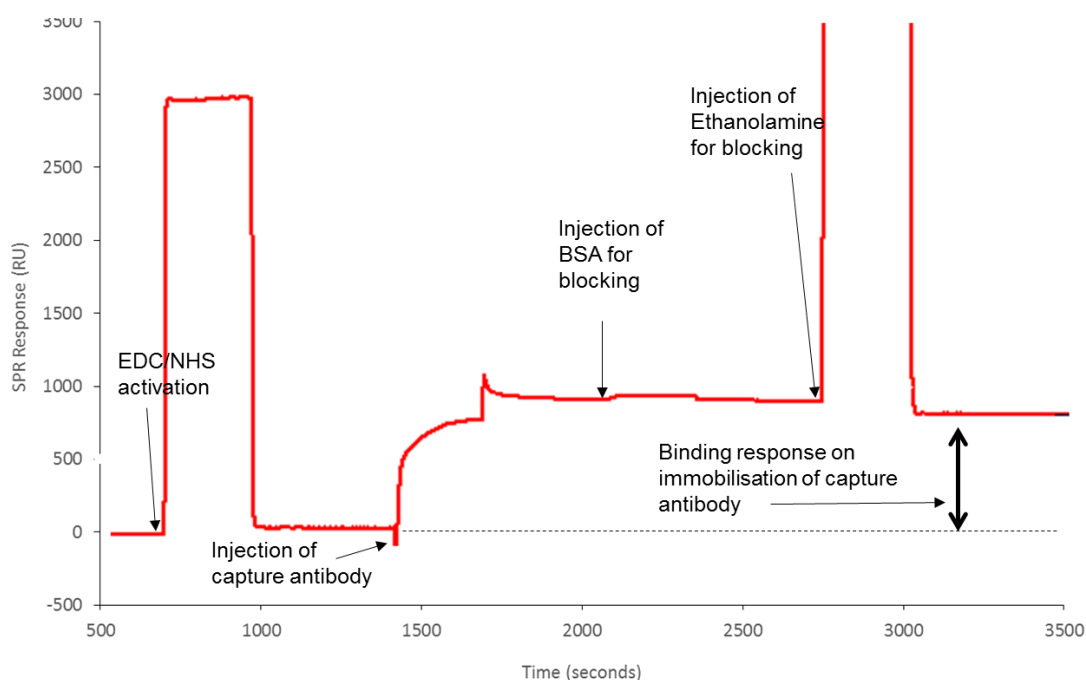
Figure 2.5: Sensorgram which shows activation of sensor chip, immobilisation of 50 μg ml^{-1} anti-cTnT 1C11 antibody (in sodium acetate buffer 10 mM, pH 4.5) followed by blocking of the sensor surface with 50 μg ml^{-1} BSA solution and 1M ethanolamine solution.

When using a small number of chips, they were frequently cleaned with piranha, and recoated with SAM. As the number of chips increased, it was observed that the antibody binding responses obtained on immobilisation were somewhat

variable, and generally decreasing. Retrospective analysis of the antibody immobilisation binding response data for each chip prepared, found that the issue was related to the storage of the piranha cleaned chips. Although it is recommended that the cleaned chips can be stored submersed in ethanol at 4°C, this did not work when preparing cTnT immunosensors for maximum sensitivity. As shown in Figure 2.6, the immobilisation responses decreased with the length of time the chip was stored in ethanol after cleaning with piranha solution.
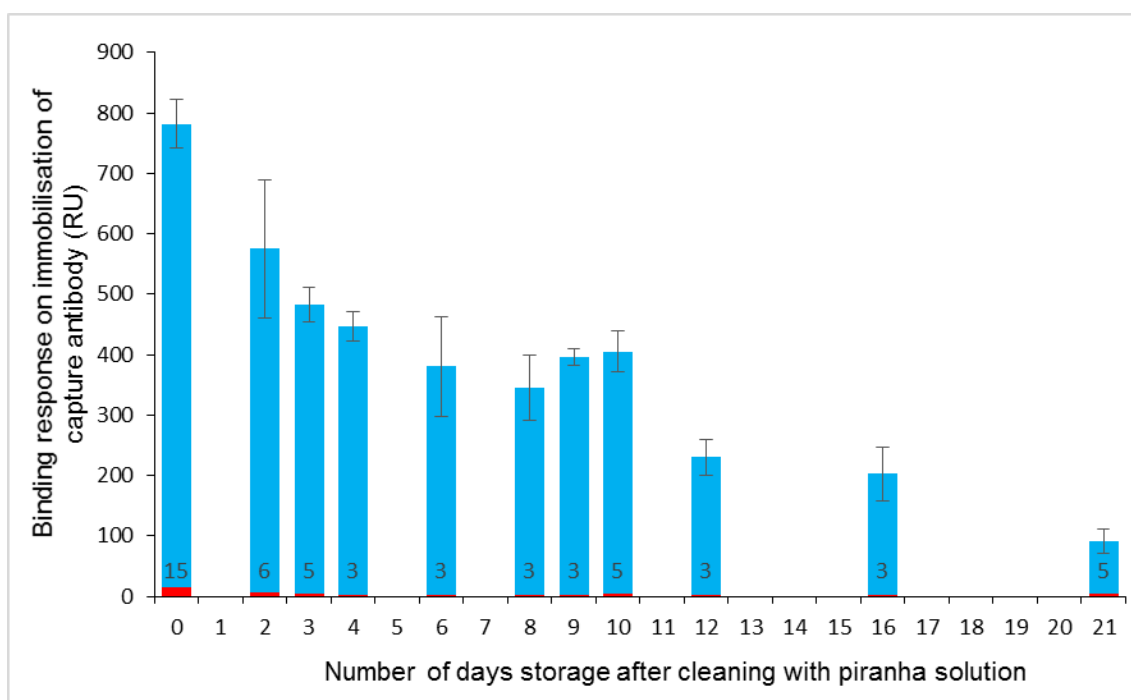
Figure 2.6: Effect of storage time (days) of piranha cleaned sensor chips prior to SAM application on immobilisation binding response of capture antibody (50 µg mL^{-1} anti-cTnT 1C11). Error bars represent SD (number of replicates for each period are specified at the bottom of each bar).

After this was determined, the sensor chip to be used was always freshly cleaned, or stored in ethanol at 4°C for a maximum of 24 hours, prior to SAM application. Alternatively, it could also be stored dry, in an airtight container. It is likely that during storage, even in ethanol, there is an opportunity for the very reactive gold binding sites to oxidise or bind to any contaminant molecules in the ethanol. This leads to fewer binding sites being available for the binding of

the 11-MUDA molecules during the SAM application, and subsequently leads less antibody being immobilised, and more non-specific binding.

2.4.4 Deposition and optimisation of the SAM

Commercially available standard amine SPR sensor chips (from Sierra Sensors, GmbH) which are already pre-coated with a proprietary SAM, were initially used for the assay development. Once these started to lose their sensitivity, they were carefully cleaned down to the bare gold surface using piranha solution and re-coated with 11-MUDA solution to form a new SAM. When the gold surface of the chip is immersed in the alcoholic 11-MUDA solution, a disorganised layer of 11-MUDA molecules forms immediately. Through the interaction of van der Waals forces these molecules slowly (hence the overnight incubation) organise themselves into a stable monolayer, as previously shown in Figure 2.1, with all the molecules orientated in the same direction, the carboxyl groups facing outwards. These recoated chips can then be used for further experiments.

The optimum concentration of 11-MUDA for coating the sensor chip was investigated by testing concentrations of 2, 4 and 10 mM 11-MUDA in ethanol. The tests were performed by applying the SAM layer, immobilising the capture antibody, blocking any remaining active sites, and then injecting the cTnT solution and measuring the SPR binding response. The results of the preliminary binding test for each individual chip were collected and compared, as shown in Table 2.2.

Table 2.2: Effect of 11-MUDA concentration used for SAM on cTnT binding responses obtained for different concentrations of cTnT.

Concentration of cTnT (ng mL^{-1})	SPR Response (RU)						Mean	SD	CV (%)
	2 mM 11-MUDA		4 mM 11-MUDA		10 mM 11-MUDA				
	1st chip	2nd chip	1st chip	2nd chip	1st chip	2nd chip			
250	132.6	129.5	141.5	125.6	125.3	(68.2[a])	130.9	6.6	5.0
500	333.6	331.7	333.6	321.7	261.7	(115.5[a])	316.5	31.0	9.8
1000	738.7	653.8	759.5	636.5	696.9	(209.4[a])	697.1	52.8	7.5

a   Not used in statistical calculations (as there appears to have been a problem with this chip)
SD; standard Deviation, CV; Coefficient of Variation

The data show that there was little difference between the different concentrations of 11-MUDA used, with respect to the effectiveness (as shown by the cTnT binding response) of the SAM coating. These data therefore suggest that a 2 mM concentration is sufficient to fully coat the sensor surface. From investigations conducted later in the development of the immunosensor, the reduced cTnT binding response for the second chip at the 10 mM concentration may have been due to the increased time the cleaned chip had been stored, prior to application of the SAM (as detailed in section 2.4.3).

2.4.5 Optimisation of SPR running buffer

The preliminary binding experiment described in section 2.3.6 using three different concentrations of cTnT was first conducted using PBS as the running buffer for the SPR instrument. The sensor surface had been immobilised with 50 µg mL^{-1} anti-cTnT 1C11 capture antibody, then three different concentrations of cTnT were injected and the binding response measured. This experiment was then repeated using PBS/T added as the running buffer, and the two sets of data obtained were compared.

The response obtained on injection of cTnT was nearly doubled when 0.05% Tween 20 was added to the running buffer, as shown in the sensorgram in

Figure 2.7, and the bar chart in Figure 2.8. This increase in response was the same for the three different concentrations of cTnT tested. This suggests that there may be some non-specific binding of the cTnT to the flow-path within the SPR. The addition of the detergent Tween 20 reduces the non-specific binding resulting in more cTnT being available to bind to the capture antibody immobilised on the sensor surface.

1000 ng mL^{-1} Troponin T
RB = PBS + 0.05% Tween 20

1000 ng mL^{-1} Troponin T
RB = PBS only

250 ng mL^{-1} Troponin T
RB = PBS + 0.05% Tween 20

250 ng mL^{-1} Troponin T
RB = PBS only
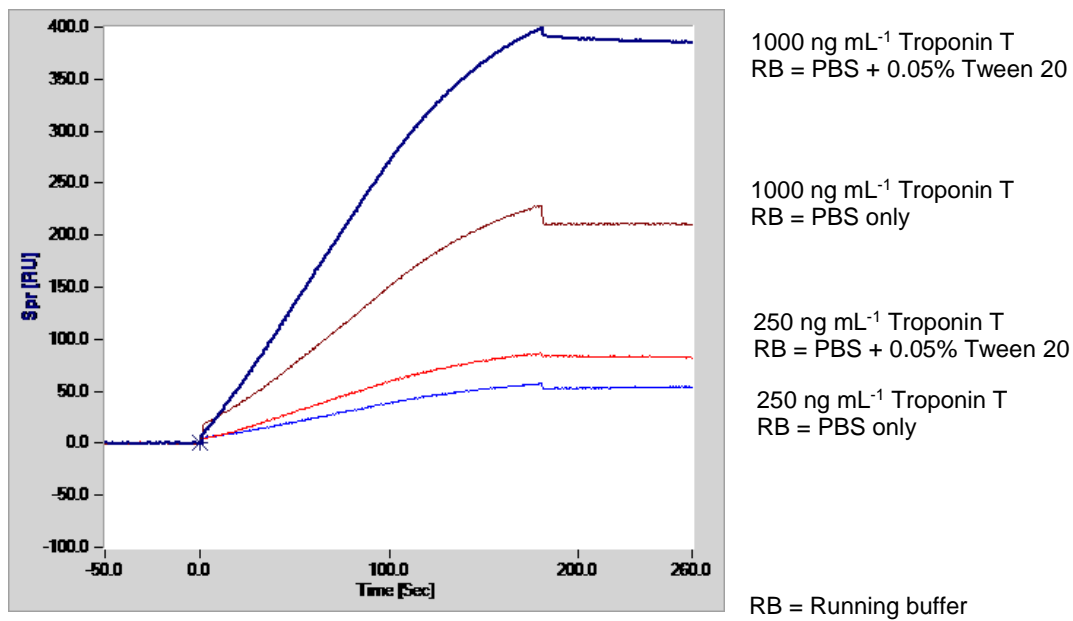
RB = Running buffer

Figure 2.7: Sensorgram showing effect of detergent (Tween 20) on binding of cTnT. One set of samples were run with and without 0.05% Tween 20 added to the PBS running buffer, details as for Figure 2.7.
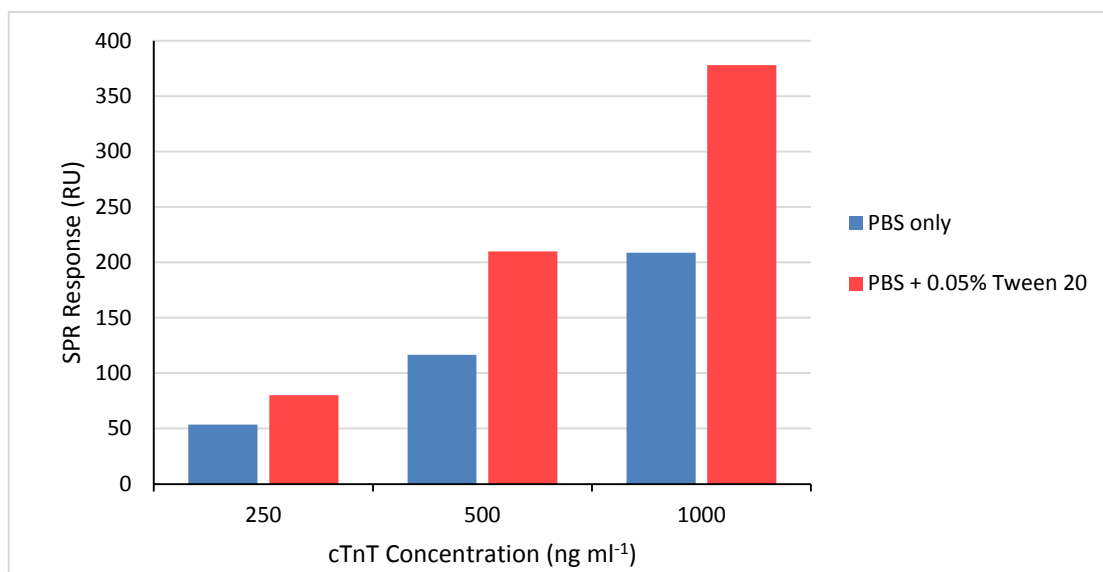
Figure 2.8: Comparison of responses for binding of cTnT with and without 0.05% Tween 20 added to the PBS running buffer. 50 µg mL^{-1} of anti-cTnT 1C11 antibody immobilised on sensor surface, 250, 500 and 1000 ng mL^{-1} cTnT solutions prepared in PBS, injected for 3 minutes at 25 µL min^{-1}.

2.4.6 Direct immunoassay for detection of cTnT

A direct immunoassay was set up for the detection and quantitation of cTnT. A calibration curve was prepared using cTnT diluted in running buffer (PBS-T) to give seven calibration standards ranging from 50 to 1000 ng mL^{-1}. The sequence of calibration standards was injected (in ascending order of concentration) three times to give replicate response values which could be analysed for linearity and reproducibility. In the third series of injections, an extra calibration standard was added at a concentration of 25 ng mL^{-1} of cTnT, to explore the potential limit of detection (LOD).

Figure 2.9 shows the sensorgrams of the direct binding of cTnT to the immobilized capture antibody over the entire calibration range 50 to 1000 ng mL^{-1}.
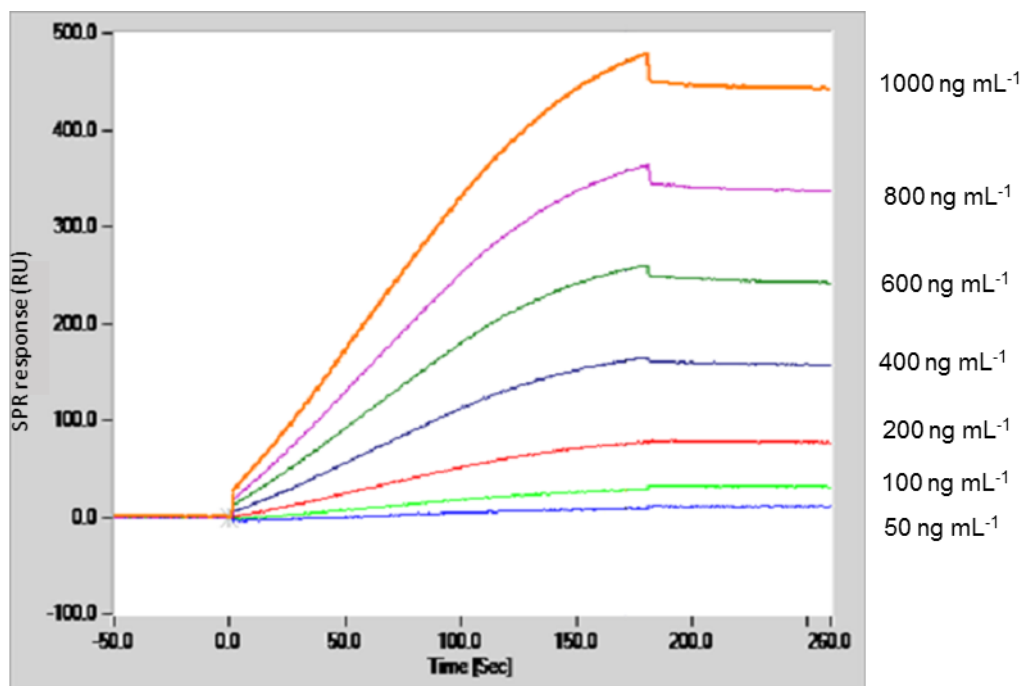
Figure 2.9: Sensorgram of direct binding of cTnT samples 50 – 1000 ng mL^{-1} (prepared in PBS/T buffer) onto anti-cTnT 1C11 antibody immobilised at a 50 µg mL^{-1} concentration. Injection time = 3 min, dissociation time = 30 sec.

The calibration curve obtained with cTnT binding to the capture antibody immobilised on the surface of the sensor chip is shown in Figure 2.10. The relationship between the mean SPR response and the concentration of cTnT bound has been shown to be very linear. The limit of detection for the assay was 26.6 ng mL^{-1} cTnT, as calculated from extrapolation three times the SD of the blank signal (obtained by injecting blank sample buffer). This was confirmed by the injection of a 25 ngmL^{-1} cTnT solution. However, the current LOD of the direct assay (26.6 ng mL^{-1} cTnT), indicates that the sensitivity of the assay will be insufficient for detection of cTnT at the required levels (<0.06 ng mL^{-1}) in serum (Apple et al., 2012). This led to the investigation of the sandwich assay format for the immunoassay to improve the response obtained.
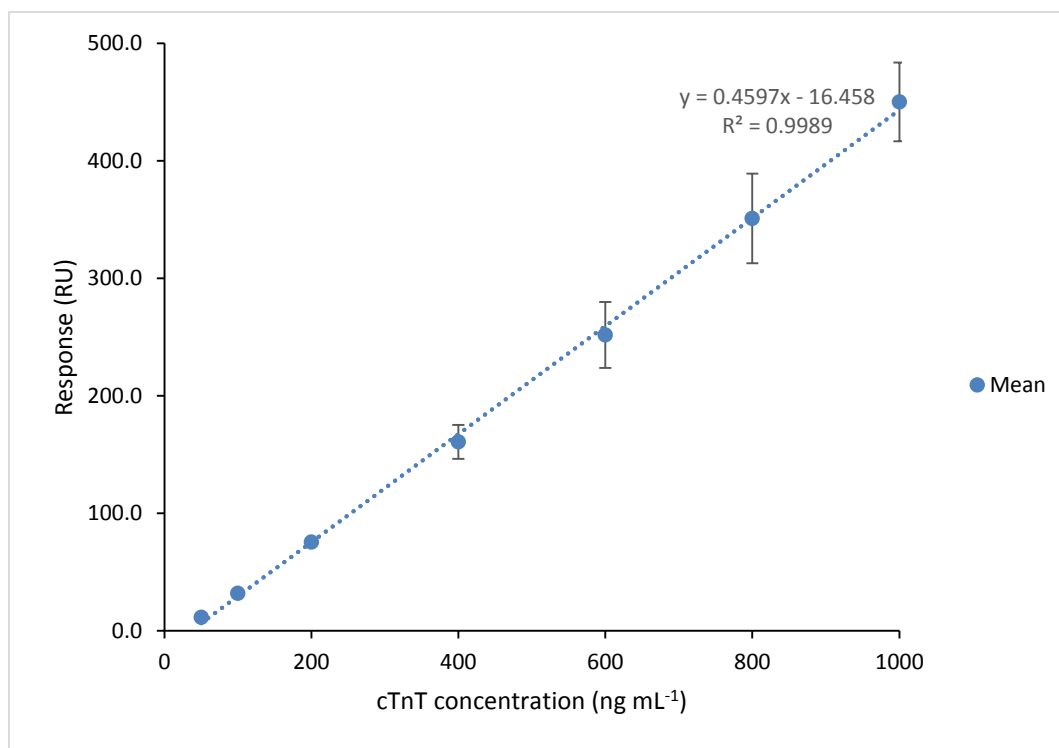
Figure 2.10: Calibration curve of mean cTnT binding response against cTnT concentration for direct assay50 µg mL^{-1} anti-cTnT 1C11 antibody immobilised, cTnT solutions (50 – 1000 ng ml^{-1}) injected for 3 min, at 25 µL min^{-1} flow rate. Each error bar represents SD (n=3). The CV ranged from 2.7 to 11.1%.

2.4.7 Sandwich immunoassay for detection of cTnT

To enhance the sensitivity of the assay by increasing the binding response, a sandwich assay format was developed using another anti-cTnT antibody, as the detection antibody. The capture antibody (anti-cTnT 1C11 antibody) specifically binds to the 95 to 181 amino acid epitope region of cTnT (Abcam datasheet, 2014a), whereas the detection antibody (anti-cTnT 7G7 antibody) specifically binds to the 60 to 70 amino acid epitope region of the cTnT molecule (Abcam datasheet, 2014b), ensuring increased specificity for the assay and reducing steric hindrance during binding.

To evaluate the signal enhancement, a fixed concentration of cTnT (200 ng mL^{-1}) was first injected for 3 minutes then and allowed to dissociate for 30 seconds. A 20 µg mL^{-1} solution of the detector antibody was then injected

for 3 minutes, and allowed to dissociate for 30 seconds. The binding response for each injection measured, and the amount of signal amplification was calculated. The sensor surface was then regenerated by injecting 0.1M HCl and 20 mM NaOH solutions for one minute each. The sensorgram in Figure 2.11 illustrates the injection sequence used.

The data presented in Table 2.3, shows that response for the binding of 200 ng mL^{-1} cTnT is amplified nearly 700% on injection of the detection antibody.

Table 2.3:   Amplification of response after binding of 200 ng mL^{-1} cTnT, followed by 20 μg mL^{-1} anti-cTnT (7G7) detector antibody.

Concentration of cTnT (ng mL^{-1})	SPR response (RU) after injection of;		Percentage Amplification
	cTnT	Detection antibody	
200	42.9	338.5	689
	42.2	324.9	670
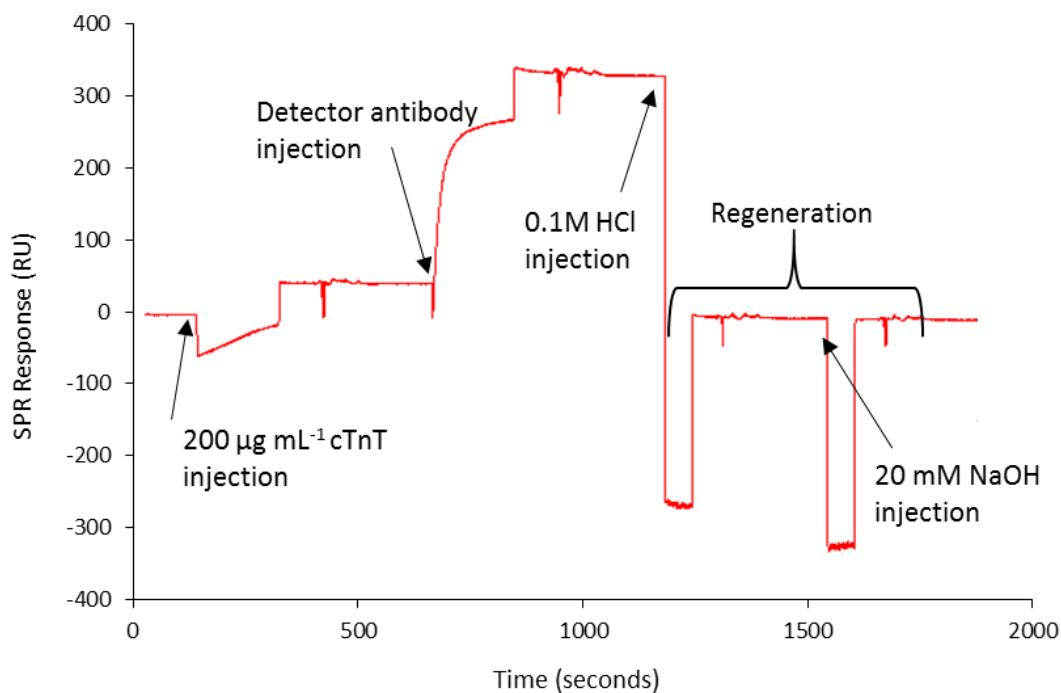
Figure 2.11: **Sensorgram of binding of 200 ng mL^{-1} of cTnT, followed by binding of 20 μg mL^{-1} anti-cTnT (7G7) detector antibody, then regeneration of sensor surface. Sensor surface already immobilised with 50 μg mL^{-1} capture antibody. cTnT and detector antibody each injected for 3 min, 0.1M HCl and 20 mM NaOH injected for 1 min each, flow rate 25 μL min^{-1}, PBS/T buffer.**

2.4.8 Optimisation of detection antibody concentration

After it was confirmed that the sandwich assay format greatly improved the sensitivity of the immunoassay, the next step was to optimise the concentration of the detection antibody used. This was achieved by injecting fixed amounts of antigen (100 ng mL^{-1} cTnT) each time, but testing different concentrations of detection antibody over a wide concentration range (1 to 16 μg mL^{-1} anti-cTnT 7G7 antibody).

Figure 2.12 shows the increased response for the sandwich assay compared to the direct binding of cTnT at a fixed concentration (100 ng mL^{-1} cTnT). The increase in response rises proportionately with detection antibody

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concentration, up to about 4 µg mL^{-1} of detection antibody. The rate of response increase then reduces as the remaining binding sites are saturated.
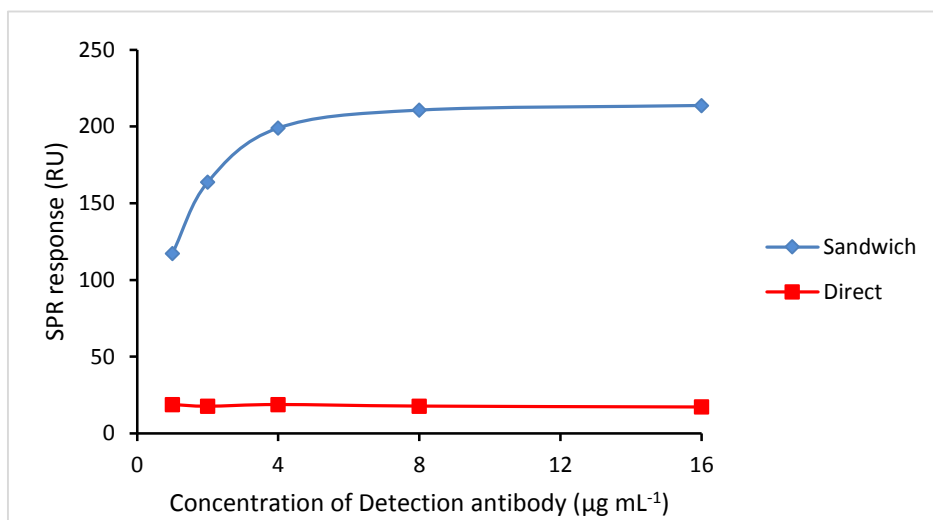
Figure 2.12: SPR Response for binding of detection antibody after initial binding of 100 ng mL^{-1} of cTnT (fixed concentration). Concentration range of anti-cTnT 7G7 antibody tested 1 to 16 µg mL^{-1}. Sensor surface already immobilised with 50 µg mL^{-1} capture antibody. cTnT injected for 3 min followed by injection of detector antibody for 3 min, followed by sensor surface regeneration (0.1M HCL for 1 min, and 20 mM NaOH for 1 min), flow rate 25 µL min^{-1}, PBS/T buffer.

The optimisation experiment was repeated using a fresh sensor chip, and a narrower concentration range of detection antibody; 1, 2, 3, 4 and 5 µg mL^{-1}, to pinpoint the optimal concentration. A similar profile was observed as shown in Figure 2.13, and the optimum concentration was found to be between 3 and 4 µg mL^{-1} of detection antibody. However, even though a higher amount of cTnT (200 ng mL^{-1}) was used, the response was similar to Figure 2.12 due to the actual sensor chip used for that experiment.
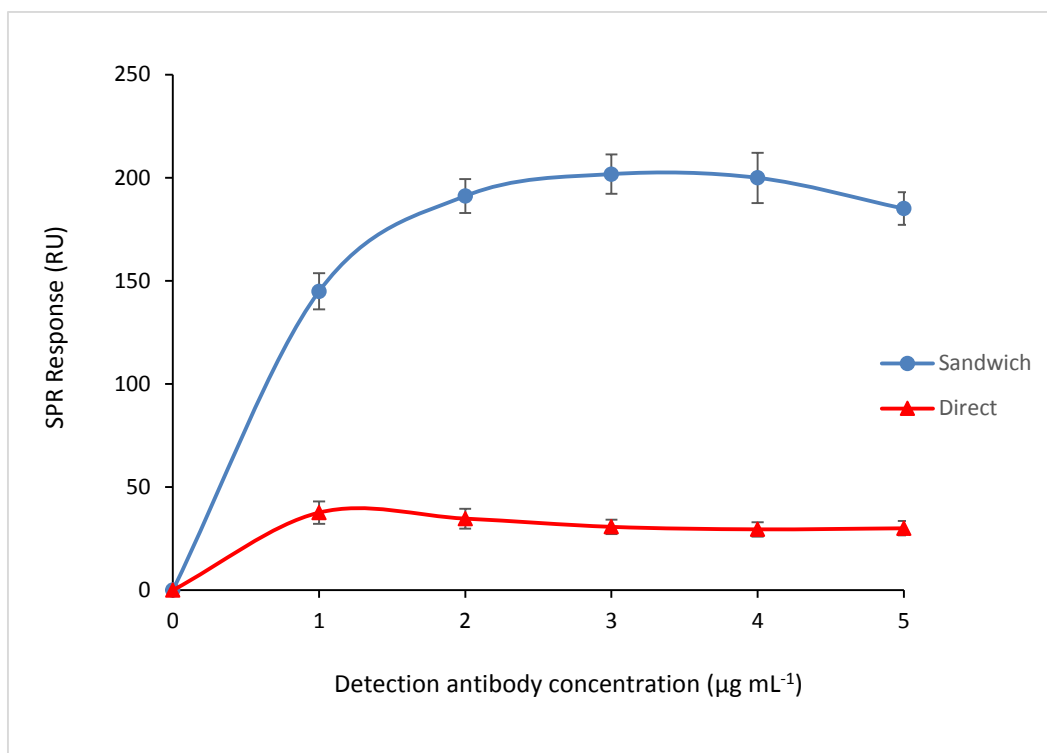
Figure 2.13: Response for binding of detection antibody anti-cTnT 7G7 after initial binding of 200 ng mL^{-1} of cTnT. Concentration range of anti-cTnT 7G7 antibody tested 1 to 5 µg mL^{-1}. Sensor surface already immobilised with 50 µg mL^{-1} capture antibody. cTnT injected for 3 min followed by injection of detector antibody for 3 min, followed by sensor surface regeneration (0.1M HCL for 1 min, and 20 mM NaOH for 1 min), flow rate 25 µL min^{-1}, PBS/T buffer. Error bars represent SD, (n=4).

Currently a rather wide calibration range of cTnT (25 – 1000 ng mL^{-1}) is being used for the early stages of this assay development. When the calibration range is reduced to the clinically expected levels (0.1 to 50 ng mL^{-1}), this optimisation will need to be repeated, further to select the optimal conditions. To make efficient use of time and progress the assay development (since a control antibody needed to be added to the assay), the LOD of the sandwich assay in PBS/T buffer was not fully assessed in replicate, and hence is not reported here.

2.5 Conclusion

This chapter describes the initial investigations and experimental work performed to develop a sensitive SPR-based immunosensor assay for the detection of cTnT. Different factors of the proposed assay procedure have been examined, and then evaluated to establish the optimum conditions.

The cardiac troponin T molecule itself is relatively small at 39 kDa in size (Dutra and Kubota, 2007a), so it can only induce a relatively small change to the refractive index when it binds to the sensor surface. Thus, a direct antigen binding assay method for cTnT would not detect the analyte at the concentration levels required, by a margin of several orders of magnitude. Hence the approach was taken to develop an immunosensor-based assay, where a capture antibody is covalently bound to the sensor surface, which in turn binds to the antigen, a direct immunoassay format. The concentration of the capture antibody immobilised, the immobilisation pH, immobilisation time and running buffer were all optimised. The preparation of the SPR sensor chip itself was also investigated, examining the optimal concentration for the application of the SAM. The preliminary results from the direct immunoassay approach indicated that the achievable LOD would be approximately 25 ng mL^{-1} cTnT. This LOD would be insufficient to develop an assay which meets the clinical need of ideally less than 0.06 ng mL^{-1} cTnT (Jaffe et al., 2012).

Next, the sandwich assay format (where detection antibody binds to the cTnT which has itself already bound to the capture antibody) was explored. The greater mass and bulk of this bound complex (39 + 160 = 199 kDa) significantly changes the refractive index, causing a measurable change in resonance angle, and so increases the SPR response. The results showed that this approach amplified the sensor signal approximately 6 to 10-fold. Assuming that this would yield an approximate 10-fold improvement in sensitivity, the LOD could be reduced down to 5 ng mL^{-1} cTnT. This is still short of the ideal LOD, so additional amplification of the sensor signal would be required. The investigations into greater signal amplification, and the further development of the cTnT immunosensor for use in human serum are described in Chapter 3.

CHAPTER 3

OPTIMISATION OF ASSAY FOR SERUM

3 OPTIMISATION OF ASSAY FOR SERUM

3.1 Introduction

Once a basic assay method has been developed, there are several issues to be considered to enable its use for the effective determination of cTnT concentrations in serum. This chapter describes the further development of the immunosensor, and its optimisation for use in human serum.

Before proceeding with further development, a control needed to be added to the assay, to monitor that the binding response being measured is due only to the cTnT antigen, and not an artefact of the method, or a different molecule binding to the sensor or antibody. The signal obtained for the control antibody is used to subtract the refractive index from the blank of the sample. The chapter will also investigate non-specific binding, which frequently occurs when protein molecules of any kind are involved. Non-specific binding is often hydrophobic in character, because the proteins compete with the water molecules, displacing them from the surface and adsorbing preferentially.

Non-specific binding can occur in several ways; the antigen could bind indiscriminately to the sensor surface (instead of specifically to the intended capture antibodies); other molecules may bind to the capture antibody, or the other molecules may bind to the antigen, which then prevent it binding to the capture antibody (usually through steric hindrance). The values for binding response obtained from the chosen control need to be subtracted from the values obtained for the target antibody binding responses, to determine the real response specific to the molecular interaction.

The non-specific binding for the developed immunosensor was investigated in both the original sample buffer and in human serum which had been diluted to different degrees. The non-specific binding and matrix interference from the serum were minimised by the addition of different additives (NaCl and BSA) to the sample buffer. However, reducing the non-specific binding often also reduces the antigen binding response as well, which will impact on the sensitivity of the assay and the limit of detection. Thus, some of the assay

parameters were re-optimised after the introduction of non-specific binding additives to the sample buffer.

When the assay had been re-optimised, it was assessed using spiked serum standards to evaluate the linearity and determine the limit of detection of the developed immunosensor, in both direct and sandwich formats.

For clinical diagnostic use a cTnT assay needs to be able to detect cTnT down to a level of 0.03 to 0.06 ng mL^{-1} (Babuin and Jaffe, 2005; Jaffe et al., 2012). Therefore the limit of detection for the developed immunosensor needed to be further reduced to achieve detection in the clinical range, which was done by employing the use of a supplementary amplification technique.

Gold nanoparticles (AuNPs) are increasingly being used for enhancing the sensitivity of biosensors, as reviewed by Saha et al., (2012). Due to their unique characteristics of biocompatibility, size- and visual properties, large surface area to volume ratios, and easy attachment of ligands, there are many methods for using them depending on the detection technique (Daree et al., 2014). They can be used with electrochemical biosensors, as they also have very good conductivity and electrocatalytic properties. The AuNPs can be deposited directly on the electrode surface, or they can be covalently bound to enzymes, DNA or immunoglobins, or carbon nanotubes; where they act by enhancing electron transfer in redox reactions, to give an amplified signal (Saha et al., 2012).

AuNPs can also be used in SPR based optical immunosensors where they can be immobilised on the sensor surface, or used in the solution phase (Bedford et al., 2012). In the solution phase they are commonly attached to an antigen or in the case of an immunosensor a secondary (detection) antibody, so they can be used for signal amplification, as shown in Figure 3.1, (Uludag et al., 2012; Li, et al 2013).
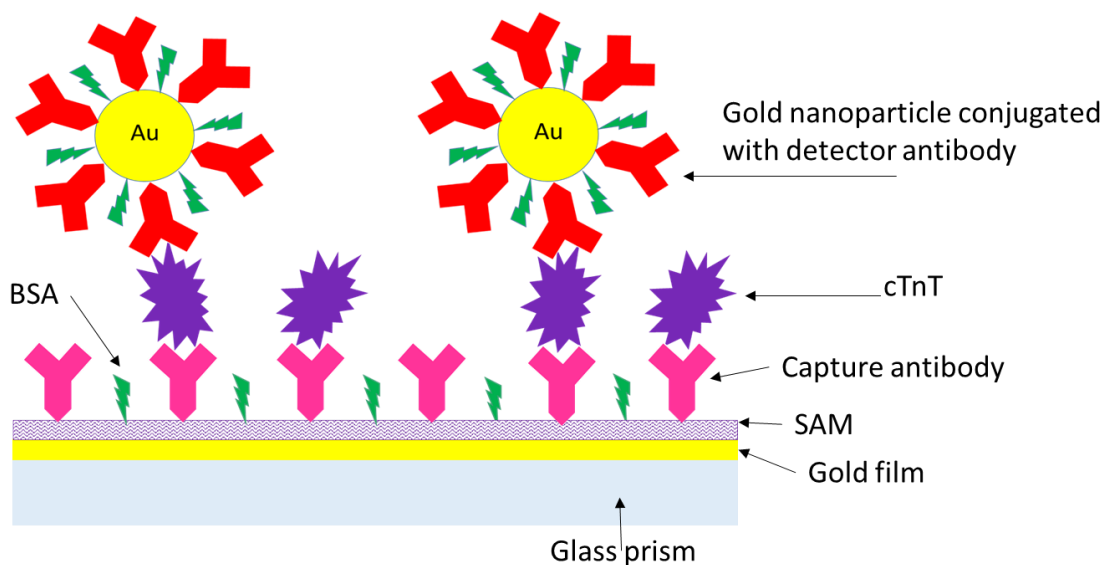
Figure 3.1: Schematic of a sandwich assay SPR based immunosensor using gold nanoparticles conjugated with detector antibody for signal enhancement.

To improve the sensitivity of the immunosensor being developed for cTnT, the amplification technique chosen to be investigated was the conjugation of the detection antibody (anti-cTnT 7G7) to AuNPs, as the sandwich immunoassay had already been optimised. Nanoparticles are usually only 1 – 100 nm in diameter, so they lend themselves to being miniaturised without affecting the function and microfluidics of devices. Therefore, once a robust immunosensor has been developed, there is the potential for it to be scaled down in size for use in a portable handheld device that can be used for diagnostics out in the field.

3.2 Materials and Equipment

3.2.1 Materials

Phosphate buffered saline (PBS, 0.01M phosphate buffer and 0.137M sodium chloride, pH 7.4) tablets, Tween 20, bovine serum albumin (BSA), sulphuric acid, ethanolamine, hydrochloric acid (HCl), dextran, hydrogen peroxide, sodium hydroxide (NaOH), sodium chloride (NaCl), bicinchononic acid reagent and copper II sulphate solution N-hydroxysuccinimide (NHS), ethanol and 11-mercaptoundecanoic acid were purchased from Sigma-Aldrich (Poole, UK).

1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) was purchased from Pierce-Thermo (Cramblington, UK). Milk diluent/blocking solution concentrate was purchased from KPL (Maryland, USA). Osmosis (RO, Ultrapure) water (18 M MΩcm-1) was obtained from a Milli-Q water system (Millipore Corp., Tokyo, Japan). The rest of the reagents were of analytical grade.

Gold nanoparticles (20 nm, 40 nm and 60 nm), Carcinoembryonic antigen (CEA) antibody, endotoxin antibody and Mouse IgG were also purchased from Sigma-Aldrich (Poole, UK). Anti-Cardiac Troponin T Capture Antibody (Mouse monoclonal (1C11) to cardiac Troponin T : ab8295), Anti-Cardiac Troponin T Detection Antibody (Mouse Monoclonal (7G7) to cardiac Troponin T : ab10223) and Cardiac Troponin T full length protein : ab9937 were all purchased from Abcam (Cambridge, UK). Human serum was purchased from Sigma-Aldrich (Poole, UK).

3.2.2 Equipment

Micropipettes were purchased from Eppendorf (Loughborough, UK). Pipette tips and 1.5 mL tubes were purchased from Fisher Scientific (Loughborough, UK). Glass ware and volumetric materials (flasks, vessels etc.) were of analytical grade. Whatman Filter paper No 1, 11 μm, was purchased from GE Healthcare Life Sciences (Little Chalfont, UK). The combined incubator/shaker for temperature controlled incubation was from Labsystem iEMS® (Labsystems, Finland), and was used with 96-well microplates from Fisher Scientific (Loughborough, UK). The rotator used for mixing was a Stuart Rotator SB3 (Bibby Scientific, Birmingham, UK). The centrifuge used was a Heraeus Fresco 17 (Thermoscientific, Loughborough, UK)

A fully automated SPR-4 biosensor, amine coated and high density amine (dextran based) chips from Sierra Sensors GmbH (Hamburg, Germany), were used to develop an immunosensor. This sensor system has four sensing spots which allow the performance of four separate assays simultaneously. All data produced by the SPR-4 were further analysed with Microsoft Excel. The spectrophotometer used for the BCA assay was a VarioskanFlash with SkanIt Software v2.4.3 (Thermoscientific, Finland).

3.3 Methods

3.3.1 Comparison of cTnT binding to target and control antibodies

The Sierra Sensors SPR-4 is a four channel SPR instrument, which allows a sensor chip to be immobilised with potentially up to four different ligands. To develop an immunosensor, one of these ligands needs to be a control antibody. An initial test was performed using an endotoxin antibody as the control antibody. The control antibody was immobilised in the same manner as the anti-cTnT capture antibody, by activating the surface with an EDC/NHS mixture, applying the antibody using a pH 5.5, 0.1M sodium acetate buffer, and then blocking the sensor surface first with BSA and then ethanolamine, as described in section 2.3.4. Standard solutions of cTnT were then injected onto the immunosensor and the binding responses measured.

Following the results which indicated the binding was the same for both the control and target antibodies, two other different potential control antibodies (mouse IgG and CEA antibody) were also tested in the same manner.

3.3.2 Investigation of the blocking step

Initial testing of potential control antibodies revealed that there was a large amount of non-specific binding taking place. Therefore, the non-specific binding was investigated in more detail to identify the type of non-specific binding that was occurring, and where it was occurring.

The first step was to check if there were still active sites on the sensor surface, by testing the efficiency of the blocking procedure. An experiment was conducted to compare blocking with milk proteins to blocking with BSA. The same volume of a 5% milk diluents solution was used in place of the BSA. A 200 ng ml^{-1} cTnT solution was injected in replicate (n=3) on each spot to assess the cTnT binding response.

3.3.3 Investigation of different reagents to reduce non-specific binding

There are several types of reagent that can help to reduce non-specific binding; detergents, salts, proteins and polymers (Moberg et al., 2013; Kyprianou et al., 2013). As a fairly high concentration of detergent (0.05% Tween 20) was already present in the PBS buffer being used to prepare the samples, it was decided to concentrate on investigating the effect of NaCl, BSA, and dextran on the non-specific binding during the cTnT binding affinity assay.

A 200 ng mL^{-1} solution of cTnT was prepared in PBS/T with different additives to potentially reduce non-specific binding of the cTnT to the surface of the chip. PBS/T was used as a control for comparison, with three different additives being 200 µg mL^{-1} BSA, 0.5M NaCl, and mixture of 0.5M NaCl and 200 µg mL^{-1} BSA. Dextran was tested briefly, but there was no difference observed, thus the results not reported.

The sample solutions of cTnT were injected in turn to both a control and an anti-cTnT antibody spot. After each injection, the spot surface was regenerated by injection of 0.1M HCl followed by 20 mM NaOH.

3.3.4 Effect of using different NaCl concentrations in the dilution buffer

The optimum combination of dilution buffer additives was investigated further to assess their effect on the linearity of the assay. The use of salt in the sample buffer was the most effective at reducing non-specific binding, but it was also found to greatly reduce the maximum binding response obtained for cTnT. As only a fixed concentration of 0.5M NaCl had been previously examined, this experiment was repeated using 0.25M NaCl in the sample buffer. This evaluated in more detail how the NaCl content affects both the cTnT binding and the non-specific binding.

A set of seven calibration samples in the range 25 to 400 ng mL^{-1} cTnT were prepared in each sample buffer. Buffer A consisted of 0.5M NaCl with 200 µg mL^{-1} BSA, and Buffer B consisted of 0.25M NaCl with 200 µg mL^{-1} BSA.

The samples were injected in replicate onto two different spots, one immobilised with control antibody (CEA), and one with the target antibody (anti-cTnT 1C11).

3.3.5 Assessment of linearity using non-specific binding additives in the dilution buffer in both direct and sandwich assays

A sensor chip was immobilised on three spots with anti-cTnT 1C11 antibody, and control (CEA) antibody on the fourth spot. A set of calibration standard samples across the calibration range of 25 to 400 ng mL^{-1} cTnT for the direct assay and 10 to 200 ng mL^{-1} for the sandwich assay, were prepared in non-specific binding (NSB) sample buffer (0.5M NaCl and 200 µg mL^{-1} BSA in PBS/T buffer). For the direct assay each cTnT calibration standard was injected individually onto each spot, followed by regeneration. For the sandwich assay, after the injection of the cTnT, a fixed amount of detection antibody (2 µg mL^{-1} anti-cTnT 7G7 antibody) was injected, followed by regeneration.

3.3.6 Minimisation of matrix interference for serum samples

Different concentrations of serum sample (at 10, 40, 50 and 75%) were prepared by diluting human serum with NSB buffer. A control sample of serum was also prepared in plain PBS/T buffer (without NSB additives), for comparison. After the specified dissociation period (30 seconds) under running buffer, the sensor surface was regenerated. Each diluted serum sample was injected in triplicate.

3.3.7 Effect of NSB additives on optimisation of detector antibody concentration for serum assays

To investigate if the optimum detector antibody concentration was affected by the addition of the NSB additives to the sample buffer, the concentration of anti-cTnT 7G7 detector antibody used was re-optimised. A fixed amount of cTnT (75 µL of 100 ng mL^{-1}) was injected onto the immobilised capture antibody. This was followed by an injection of either 1, 2, 3, 4 or 5 µg mL^{-1} anti-cTnT 7G7 antibody solution, and the resulting binding responses were measured.

3.3.8 Conjugation of anti-cTnT detection antibody with gold nanoparticles (AuNPs)

The use of gold nanoparticles (AuNPs) was investigated to examine if the signal obtained via a sandwich assay could be amplified even further. The AuNPs are available in the form of a colloidal gold solution, which is stored in the dark at 4°C. The solution can be used directly, as the particles are suspended in distilled water.

Initial AuNP Conjugation Procedure

The initial conjugation procedure was as follows; a 10 µL aliquot of 2 mg mL^{-1} anti-cTnT 7G7 detection antibody and 5 µL of 0.2M NaOH (to adjust the pH) was added to 1 mL of gold nanoparticle (AuNP) solution, which was then incubated (protected from light with foil) on a rotator (Stuart SB3), at an angle of 10°, for 20 minutes at room temperature. After incubation the solution was centrifuged for 25 minutes at 8000 g, at 4°C. Immediately following centrifugation the supernatant was removed and the AuNPs were re-suspended, by adding 33 µL of 10 mg mL^{-1} BSA solution and 70 µL of PBS/T solution.

Final AuNP Conjugation Procedure

A 10 µL aliquot of 2 mg mL^{-1} anti-cTnT 7G7 detection antibody and 5 µL of 0.2M NaOH (to adjust the pH) was added to 1 mL of gold nanoparticle (AuNP) solution, which was then incubated (protected from light with foil) on a rotator, at an angle of 10°, for 1 hour at room temperature. After 1 hour, 100 µL of 10 mg mL^{-1} BSA solution was added, and the mixture incubated on the rotator for a further 20 minutes. After this period the solution was centrifuged for 25 minutes at 9000 g, at 4°C. Immediately following centrifugation, the supernatant was removed and the modified AuNPs were re-suspended, by adding 100 µL of PBS/T solution.

Antibody modified AuNPs were stored at 4°C until required for use. AuNP modified antibodies were prepared fresh weekly when required.

A simple method to check for the effectiveness of the conjugation procedure is to take a sample of the re-constituted conjugated AuNPs, and observe if it flocculates on addition of a concentrated NaCl solution. The exact procedure used was to take a 100 µL sample of the "conjugated" AuNP solution, and add 20 µL of 2.5M NaCl solution and mix.

3.3.9 Optimisation of AuNP size

Three different sizes of AuNP were tested; 20, 40 and 60 nm to find the optimal AuNP size for detection antibody functionalisation. Each size of AuNP was separately conjugated with anti-cTnT (7G7) antibody, as described above in section 3.3.8. Each size of conjugated nanoparticle was tested in a sandwich assay as described in section 2.3.9¸ to evaluate which one gave the greatest amplification of SPR response, when bound to either 25, 50 or 100 ng mL^{-1} of cTnT.

3.3.10 Confirmation of AuNP conjugation

Variable results were obtained with different preparations of conjugated AuNPs, so the conjugation procedure was investigated with an extra method, in addition to the flocculation test.

The protein content of the AuNP conjugated antibodies was investigated, by using the bicinchoninic acid (BCA) method to determine the concentration of protein present after the conjugation procedure. The BCA assay only measures total protein content and is unable to differentiate between the protein from antibody binding and the protein from the BSA binding. Therefore two samples of conjugated antibody were prepared in parallel; sample A was centrifuged immediately after the incubation with AuNPs step, whilst sample B was taken through the remainder of the procedure and incubated with BSA to block any remaining active sites. Aliquots of both the supernatant and the reconstituted product/solution for each sample were analysed for protein content.

Protein calibration standards at eight different concentrations in the range 5 to 250 µg mL^{-1} were prepared by diluting a 1 mg mL^{-1} solution of mouse IgG in PBS/T. A 25 µL aliquot of either blank, control, standard or unknown sample in

the wells of a 96-well microtitre plate. All samples were analysed in triplicate if possible.

Active BCA reagent was prepared by mixing 200 µL of BCA solution with 10 mL of copper II sulphate solution. A 200 µL aliquot of active BCA reagent was added to each well. The plate was then incubated at 37°C for 2 hours in an incubator with a mild shaking capability, so enabling the samples to be mixed. The absorption of samples at the wavelength of 562 nm (due to the reaction colour change from green to purple) was determined colorimetrically, using a UV/Fluorescence spectrophotometer.

3.3.11 Assessment of the final assay method using serum

Many different parameters were investigated throughout the project which could improve the sensitivity of the proposed immunosensor. The optimum conditions ascertained through the development process were combined in the final sandwich assay method.

A fresh sensor chip (Sierra Sensors Standard amine) was used to prepare the immunosensor. The anti-cTnT 7G7 detector antibodies were conjugated with 40 nm AuNPs, by incubating them together for an hour, prior to adding a high concentration of BSA solution, and incubating for a further 25 minutes. The incubation mixture was then centrifuged, the supernatant removed, and the conjugated antibodies were reconstituted.

The final assay method was used to prepare calibration samples in the range 0.5 to 40 ng mL^{-1} cTnT, by spiking appropriate known amounts of cTnT into blank human serum which had been diluted 1:1 with NSB additive modified PBS/T buffer. These calibration standards (along with a blank serum sample) were thoroughly mixed and injected in turn onto the sensor chip, which had been freshly immobilised with 50 µg mL^{-1} of anti-cTnT 1C11 antibody on three sensor spots, and control antibody (CEA), on the fourth position. The assay was run twice, once with unmodified antibodies for detection, and once with the AuNP conjugated detector antibodies, and the results compared.

3.4 Results and discussion

It is very difficult to measure biomarkers such as troponin in "real time" in undiluted serum samples, due to the extremely low concentrations of troponin and the very high and varied amounts of serum proteins. This is largely due to matrix interference from the serum proteins, and their adsorption to sensor surfaces (Nath et al., 2004).

SPR cannot distinguish between the binding of specific and non-specific antigens, thus an assay method needs to distinguish and avoid or at least minimise non-specific binding. There are two main stages which non-specific binding of proteins can be reduced during the development of immunosensors; during immobilisation and later during antigen binding. Employing just one strategy will rarely work, and usually a combination of both approaches has to be used.

The primary strategy for non-specific binding reduction when working with biosensors is to reduce the hydrophobicity of the sensor surface by applying a SAM to the gold layer on the sensor (Battaglia et al., 2005). Many different compounds have been investigated and used for this purpose; alkanethiols such as MUDA, 16-mercaptohexadecanoic acid and mercaptopropanol (Prime and Whitesides 1993; Love, et al., 2005; Lee, et al., 2005; Yoon et al., 2011, Stan et al., 2012) and a wide range of polymers such as OEG, PEG, poly-L-lysine and polystyrene sulphonate (Cao et al., 2006; Kurita, et al., 2006; Kyprianou et al., 2013). This is followed by amplification of the signal that is specific to the analyte or antigen being measured, so that the impact of matrix interference is reduced.

The secondary strategies then deal with the solvent environment, to further reduce the electrostatic interactions between the proteins and matrix interferences in solution and the sensor surface. The tactics that have been used include; addition of detergent (Trevino et al., 2009; Kyprianou et al., 2013), or salt (Trevino et al., 2009, Moberg et al., 2013), increasing the ionic strength of the buffer (Moberg et al., 2013) and adding additives such as BSA, protein A,

milk proteins and dextran (Uludag and Tothill 2010, Chung et al., 2006; Laguna, et al., 2014; Reichert Technologies, 2014).

Finally there are also tertiary strategies that can be employed which look at the serum samples themselves: diluting the serum until the non-specific binding from the serum proteins becomes negligible (Ramos-Jesus et al., 2011), or prior removal of the interfering proteins from the serum sample before analysis is undertaken, e.g. by heat treatment or some type of affinity chromatography (Masson et al., 2007).

Using large sample dilutions (e.g. 20 to 1000-fold) is not an option, as cTnT is present in serum at very low concentrations (0.01 to 1 ng mL^{-1}), so would be diluted by the same factor. This would raise the achievable LOD of the assay beyond the threshold level, whilst the aim of the project is to develop a highly sensitive sensor. Similarly, although removal of some types of serum protein may be possible, these are extra procedures which require extra time, whereas the aim is to develop an immunosensor that could give fast results in 15 to 20 minutes.

3.4.1 Comparison of cTnT binding to target antibodies and control antibodies

Initial tests using an endotoxin antibody as the control antibody, showed that there was a considerable amount of non-specific binding occurring, for which the control antibody was not compensating. The responses for the control were still high when compared to the responses obtained for the binding to the cTnT capture antibody. Even after optimisation, as shown in Figure 3.2 there was still a strong response obtained on the control spot, which was approximately 67% of the cTnT binding response measured for the capture antibody spots.

Different control antibodies were tested, most extensively carcinoembryonic antigen (CEA) antibody). The results were similar to that of the endotoxin antibody, indicating that there was significant non-specific binding of the cTnT protein taking place on the sensor surface. As both the endotoxin and CEA

antibodies gave similar results, CEA was chosen as the control antibody, and taken forward into the further work required to reduce the non-specific binding.
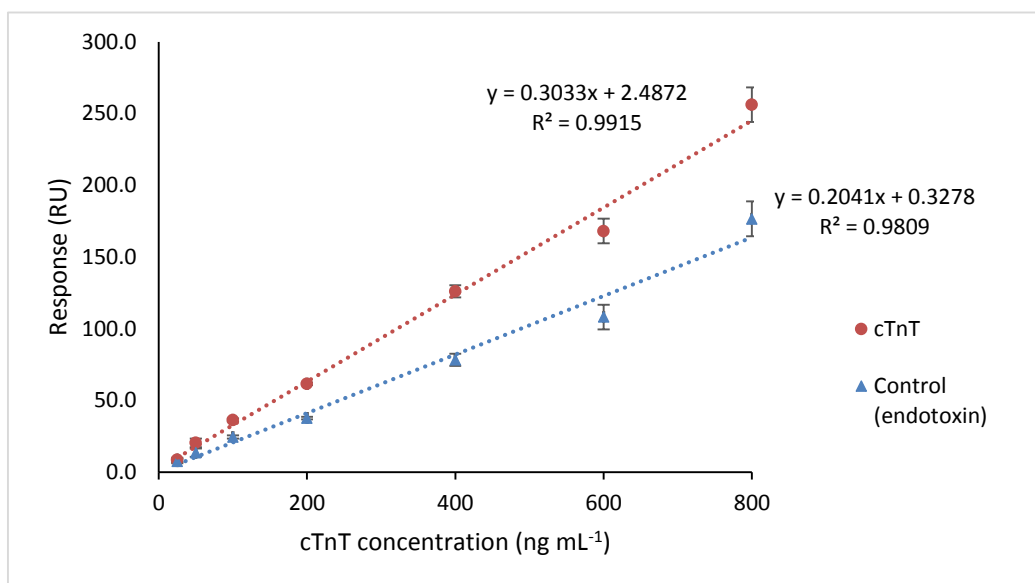
Figure 3.2: Comparison of cTnT binding responses for a sensor immobilised with capture (50 µg mL^{-1} anti-cTnT 1C11) and control (50 µg mL^{-1} endotoxin) antibodies on separate sensing spots on the sensor chip surface. Each cTnT solution (25 – 800 ng mL^{-1} in PBS/T buffer) injected for 3 minutes, followed by regeneration. Each error bar represents SD of n=3.

3.4.2 Investigating the blocking step

Milk proteins, such as skimmed milk or casein have sometimes been used as blocking agents in place of BSA (Liu et al., 2010; Laguna et al., 2014; Fakanya and Tothill 2014). Laguna et al., (2014) found that using skimmed milk proteins increased the sensitivity of their dengue virus immunosensor by 1.5 to 2 fold. Therefore, commercially prepared milk diluent (5% skimmed milk equivalent) was tested with this immunosensor, to see if it could improve blocking and so reduce non-specific binding.

The blocking of the sensor surface was examined using an increased concentration of BSA solution (100 µg mL^{-1}), but this did not reduce the non-

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specific binding of a fixed concentration (200 ng mL^{-1}) cTnT solution. The value obtained for cTnT binding was approximately 90 RU, on activated and blocked sensor surface.

The results are presented in Table 3.1, and show that there doesn't appear to be much difference between the two blocking agents, as the cTnT non-specific binding responses were very similar, with similar %CVs as well. Thus, a decision was made to keep using the BSA for consistency with previous experiments.

Table 3.1: Comparison of BSA and skimmed milk protein as blocking agents.

Blocking Agent	Mean cTnT binding response (RU)	sd	CV%
BSA (50 µg mL^{-1})	87.1	4.2	3.3
BSA (100 µg mL^{-1})	88.6	7.6	8.6
5% skimmed milk	84.1	6.5	7.7

3.4.3 Investigation of different reagents to reduce non-specific binding

Three strategies for minimising non-specific binding in this assay had already been incorporated into the method during the initial development phase; namely using a SAM (11-MUDA) to coat the sensor surface, blocking any unoccupied sites on the sensor surface with BSA and ethanolamine and the use of detergent (Tween 20) in the running and sample buffers. The combination of these strategies did not sufficiently reduce the non-specific binding, therefore the next step was to explore using other additives in the dilution buffer, to disrupt the non-specific binding.

The non-specific binding to the blocked sensor surface was examined by first activating the 11-MUDA sensor surface with EDC/NHS solution, then blocking with BSA and ethanolamine (without immobilising any capture antibodies). A 200 ng mL^{-1} cTnT solution was then injected which had been prepared in either PBS/T or PBS/T containing different additives.

Adding BSA only was shown to have no effect on reducing non-specific binding (Figure 3.3). The BSA molecules compete against the cTnT molecules for the non-specific binding sites, and as there is an excess of BSA, it will bind to the majority of non-specific sites, hence the signal increases.
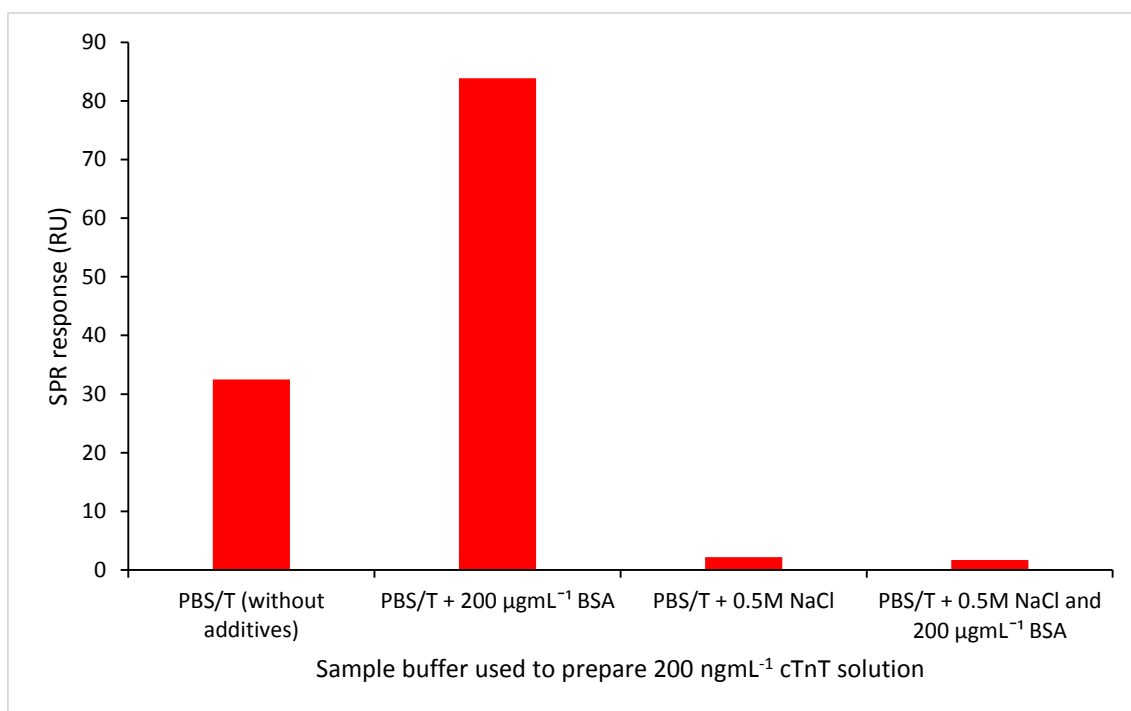
Figure 3.3: Effect of different buffer additives on the non-specific binding response of cTnT, on an activated (but not antibody immobilised) and blocked (100 µg mL^{-1} BSA and 1M ethanolamine) sensor surface. A 200 ng mL^{-1} cTnT, solution prepared in different buffers was injected for 3 minutes, with regeneration following after each injection (flow rate 25 µL min^{-1}).

The results in Figure 3.3 and Figure 3.4 show that the addition of NaCl was the most effective strategy for reducing non-specific binding in this assay. The reason for the effectiveness of NaCl is that it disturbs the electrostatic interactions by which the protein molecules bind. A NaCl concentration of 0.5M is about the highest that can be used to disrupt non-specific binding, as beyond this specific binding will also start to be disrupted (Otto and Lee, 1993). For the

cTnT molecule this disruption may have already begun, as the binding response was only about half that of when no NaCl was present. Alternatively this result could be indicating that when no additives were used the cTnT binding response was being overestimated.
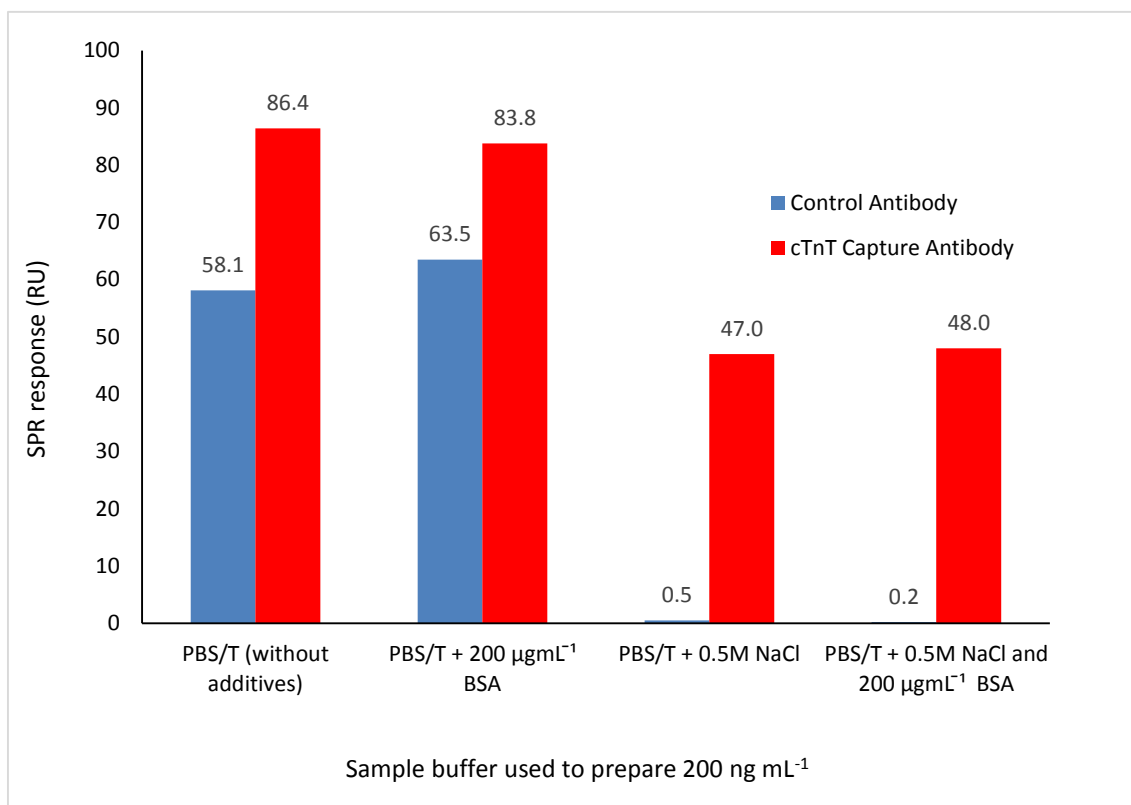
Figure 3.4: Effect of different buffer additives on non-specific binding response on sensor surfaces, which have either control or capture antibody immobilised (capture antibody = 50 μg mL^{-1} anti-cTnT 1C11; control antibody = 30 μg mL^{-1} CEA binding antibody). A 200 ng mL-1 cTnT, solution prepared in different buffers was injected for 3 minutes, with regeneration following after each injection (flow rate 25 μL min^{-1}).

Other studies have already investigated whether the addition of non-specific binding reduction additives to the running buffer or to the sample buffer is effective or not (Moberg et al., 2013). The conclusion from the studies of Moberg et al., was that it makes no difference to which buffer the additives are added (it is the total ionic concentration which is important), and the effects are not cumulative if they are added to both.

3.4.4 Effect of NaCl concentration in the sample buffer on cTnT binding response

Previous results had shown that using a 0.5M NaCl concentration in the sample buffer appeared to eliminate non-specific binding to the control antibody, but unfortunately also reduced the overall cTnT binding response as well.

Higher concentrations of NaCl were not investigated, as firstly, these results indicated there was no need to increase the concentration further (as non-specific binding on the control antibody had been reduced to near zero), and secondly as shown in Figure 3.5, the binding response for cTnT was being reduced, so it would be difficult to obtain the sensitivity required for the assay if the cTnT response was decreased further.
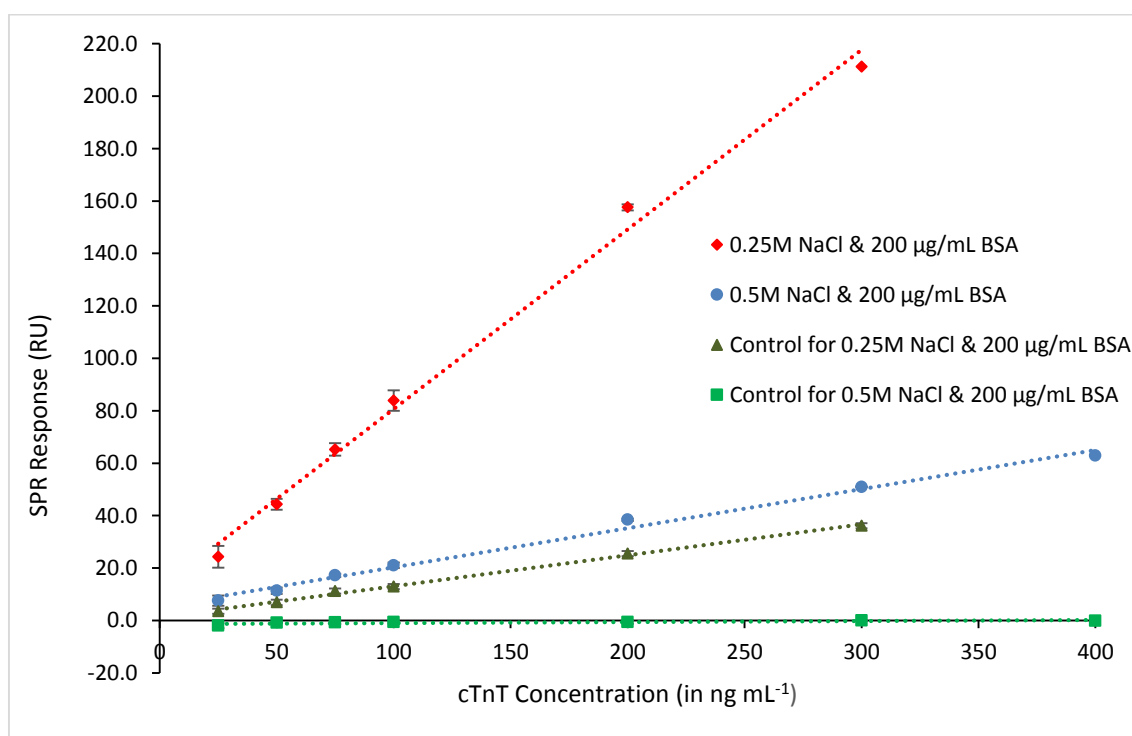
Figure 3.5: Effect of different NaCl concentrations in sample buffer on cTnT binding response to capture and control sensing spots. Sensor surface spots immobilised with 50 µg mL^{-1} anti-cTnT 1C11 or 30 µg mL^{-1} control antibody (CEA). Calibration solutions of cTnT (25 – 400 ng mL^{-1}), prepared in dilution buffer (either 0.25 or 0.5M NaCl + 200 µg mL^{-1} BSA) were injected for 3 minutes, with regeneration following after each injection (flow rate 25 µL min^{-1}). Each error bar represents SD of n=3.

A 0.25M NaCl concentration was investigated to see if a lower NaCl concentration would still prevent non-specific binding, whilst improving the cTnT binding response. By halving the NaCl concentration, the binding response was shown to be four times greater at cTnT concentrations of 200 ng mL^{-1} and below. If the assay was only to be used with solutions, using 0.25M NaCl would be viable way to get better sensitivity. However, since this assay is intended for use with serum, it was likely that using serum would increase the protein load and cause additional signal. Thus, it was decided to continue using the higher 0.5M NaCl concentration which managed to eliminate the background signal for the cTnT assay.

3.4.5 Assessment of linearity using non-specific binding additives in the dilution buffer in both direct and sandwich assays

The effect of the NSB buffer additives on the linearity of binding response was investigated using calibration standard samples (prepared in non-specific binding (NSB) dilution buffer) across the calibration range of 25 to 400 ng mL^{-1} cTnT for the direct assay and 10 to 200 ng mL^{-1} for the sandwich assay. For the direct assay each cTnT calibration standard was injected individually onto each spot, followed by regeneration. For the sandwich assay, after the injection of the cTnT, a fixed amount of detection antibody (2 μg mL^{-1} anti-cTnT 7G7 antibody) was injected, followed by regeneration.

The results show that the NSB additives do not affect the linearity of the assay in either the direct or sandwich assay format (Figure 3.6). The non-specific binding (NSB) dilution buffer used (0.5M NaCl and 200 μg mL^{-1} BSA in PBS/T buffer) was effective at eliminating non-specific binding for the solutions tested as shown by the results for the control antibody. The use of the additives does however reduce the binding response for cTnT overall. This in turn affects the maximum achievable binding response for cTnT when using the sandwich assay, because less cTnT is available for the detector antibody to bind to. Overall the response for the sandwich assay was amplified by a factor of 3.3 fold, compared to the direct assay. The LOD achieved for the direct assay was 16.9 ng mL^{-1} cTnT, and for the sandwich assay the LOD was 4.59 ng ml^{-1}.
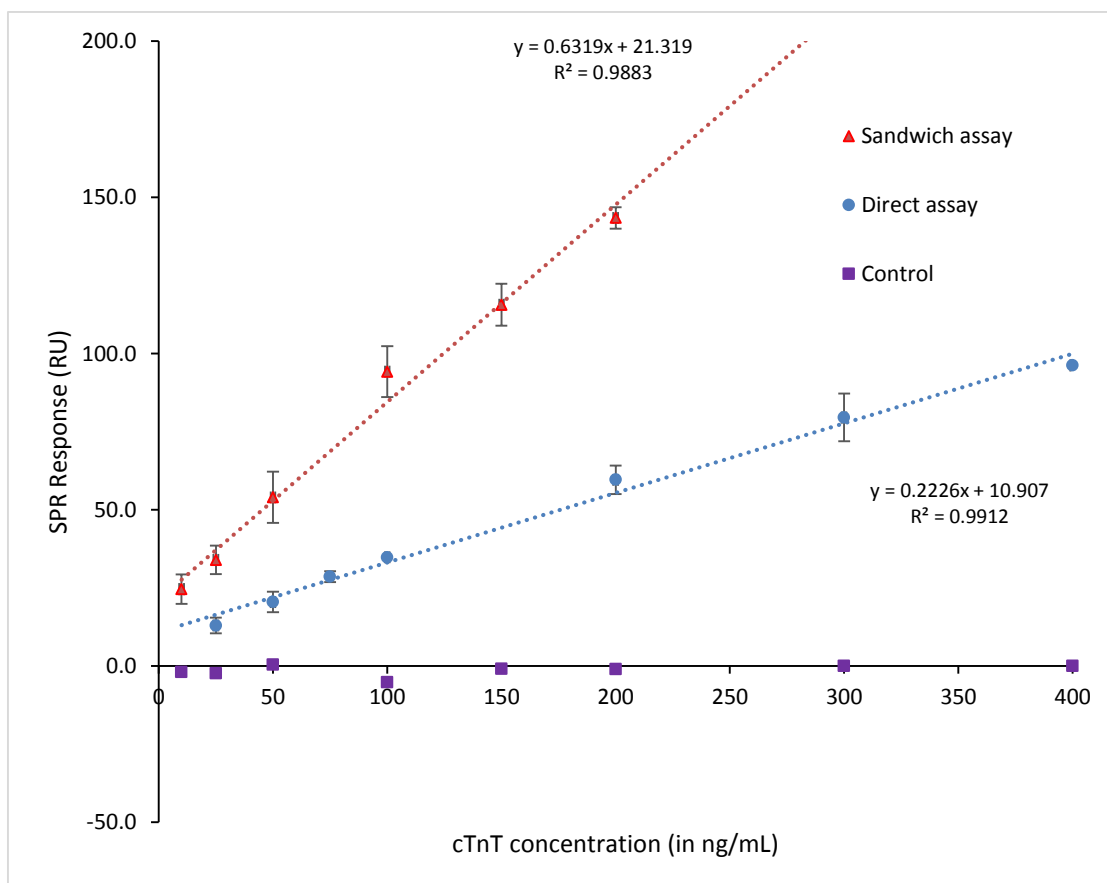
Figure 3.6: Plot of cTnT concentration against sensor signal for direct and sandwich assay of cTnT. Sensor surface spots immobilised with 50 μg mL⁻¹ anti cTnT 1C11 or 30 μg mL⁻¹ control antibody (CEA). Calibration solutions of cTnT (25 – 400 ng mL⁻¹), prepared in dilution buffer (0.5M NaCl + 200 μg mL⁻¹ BSA in PBS/T) were injected for 3 minutes (flow rate 25 μL min⁻¹). Direct assay sensor spots regenerated after cTnT binding, sandwich assay sensor spots regenerated after detector antibody binding. Each error bar represents SD of n=3.

3.4.6 Minimisation of matrix interference for serum samples

Serum contains many different molecules such as proteins, enzymes, carbohydrates, lipids and ions, which can all contribute to matrix interference issues. The largest and most problematic component is proteins, as the normal range for total protein content in serum is 63 to 80 mg ml^{-1} (FDA, 2014). Thus, the next step in the assay development was to assess how effectively the NSB buffer additives prevented non-specific binding of serum proteins when actual human serum was injected.

Figure 3.7A shows the sensorgram obtained for injections of different dilutions of serum, with the regeneration injections in between. The bulk transport effect is very large because of all the serum proteins affecting the refractive index, thus the changes in SPR signals are difficult to see because of the scale of the y-axis. Thus, in Figure 3.7B, the y-axis of the sensorgram has been expanded, and shows more clearly the binding response due to non-specific binding of serum proteins.

(A)
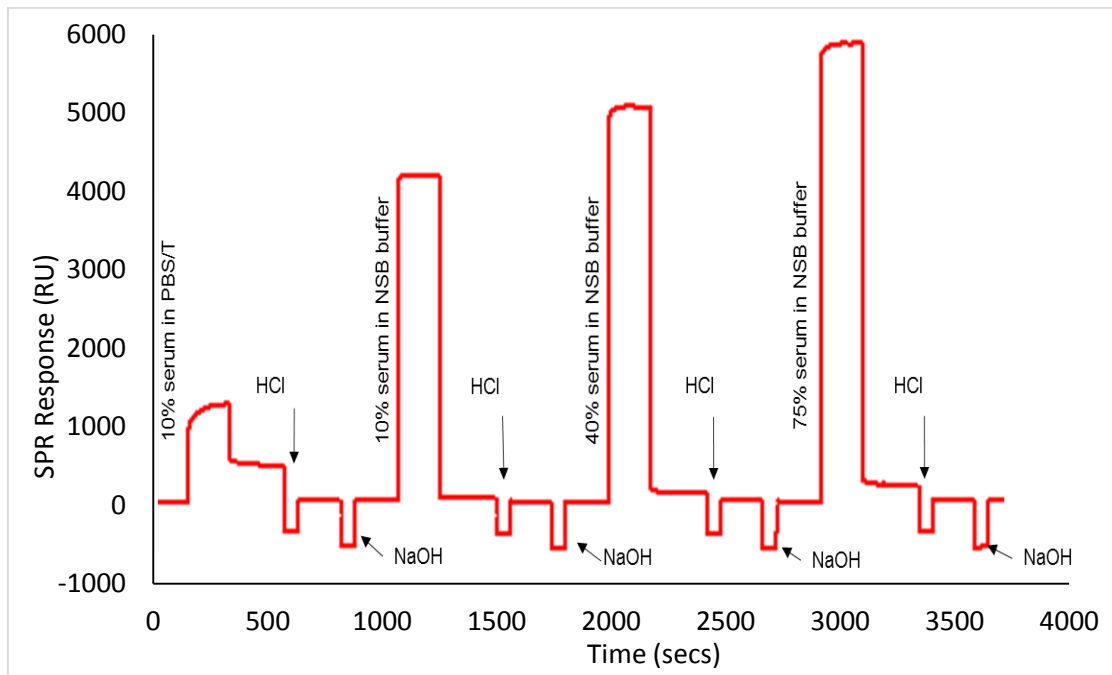
(B)
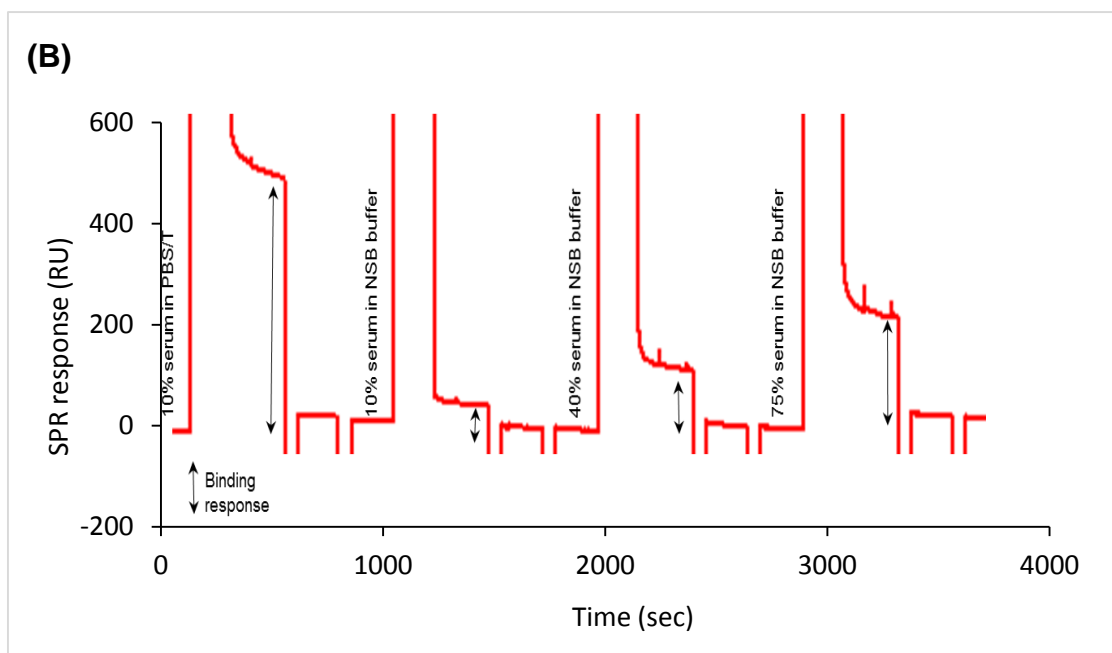
Figure 3.7: A) Sensorgram of non-specific binding of different concentrations of human serum in PBS/T buffer, and PBS/T buffer with NSB additives added to dilution buffer. B) Expanded version of sensorgram (A), showing the binding responses in greater detail for increasing concentrations of human serum. Sensor surface immobilised with 50 µg mL^{-1} anti-cTnT 1C11 antibody. Binding injections were 3 minutes at flow rate of 25 µL min^{-1}, followed by regeneration.

The non-specific binding results obtained for diluted serum (10%, 40%, 50% and 75%) are shown in Figure 3.8. A comparison of the results obtained for serum diluted either 10% in the PBS/T running buffer or 10% in the NSB buffer, shows the non-specific binding is reduced from 442 RU to 30.6 RU, when the NSB additives are used. This is a 93% reduction in the original amount of non-specific binding, which demonstrates the effectiveness of the NSB additives. As the percentage of serum in the sample is increased (and therefore the volume of NSB buffer added is reduced), the non-specific binding begins to increase again. Since there is little increase in non-specific binding when the serum concentration is increased from 40 to 50%, and a 1 to 1 dilution is simple to carry out (e.g. if a immunosensor is used in a location, with little access to laboratory equipment), it was decided to use a 50% serum dilution for further investigations of this cTnT assay. This compromise of performing a simple dilution to reduce non-specific binding when using serum has also been made by others (Trevino et al., 2009; Luo, et al., 2013; Dong, et al., 2014).
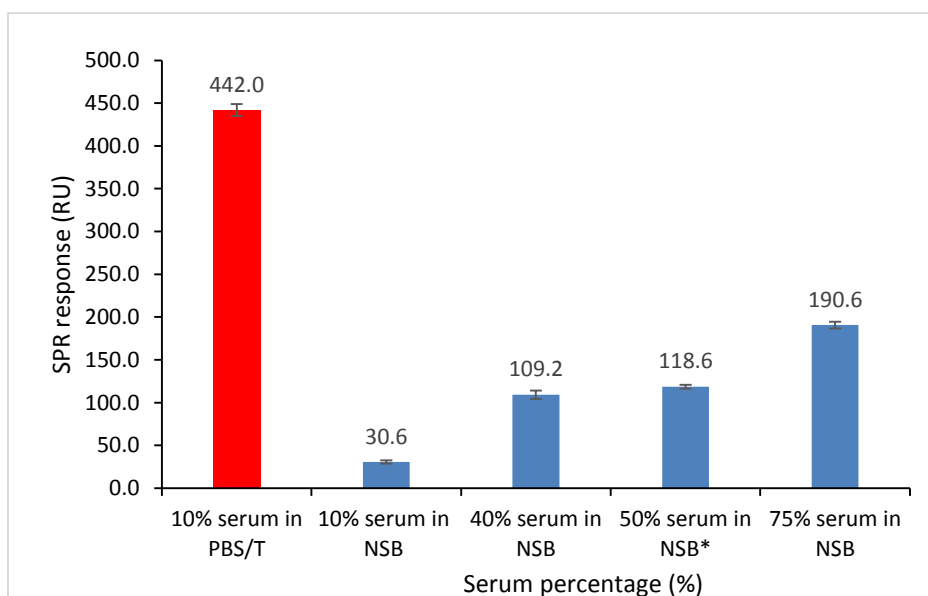
Figure 3.8: Effect of increasing human serum concentration on the sensor signal, in PBS/T sample buffer with NSB additives compared to PBS/T buffer only. Sensor surface immobilised with 50 µg mL^{-1} anti-cTnT 1C11 antibody. Binding injections were 3 minutes at flow rate of 25 µL min^{-1}, followed by regeneration.

3.4.7 Effect of NSB additives on optimisation of detector antibody concentration for serum samples

Following the investigation into non-specific binding, with the subsequent change of using of NSB additives in the dilution buffer, there was a need to re-optimise the concentration of the detector antibody used for the sandwich assay. Although minimising the non-specific binding, the additives also affected the overall binding response for cTnT, thus it was likely that less cTnT was being bound to the capture antibody. If the cTnT concentration bound was less, or if the extra NaCl or BSA were hindering binding of the secondary antibody, the optimum concentration of anti-cTnT 7G7 antibody for signal amplification may have also changed, and so was examined again. The results are shown in Figure 3.9.
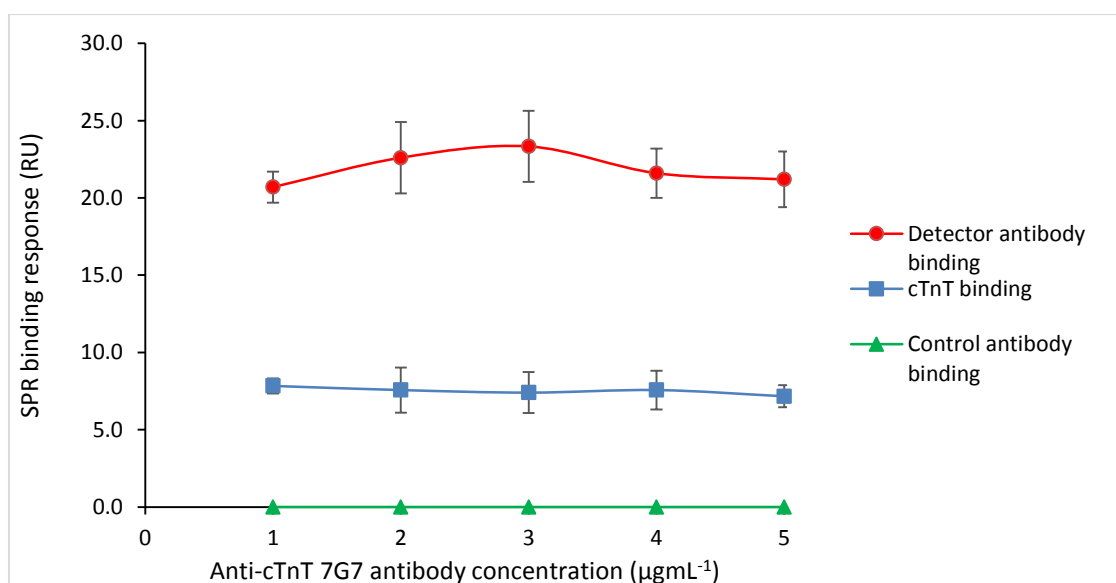
Figure 3.9: Plot of binding responses for cTnT (both direct and amplified via sandwich assay) against detector antibody concentration, for both control and cTnT spots. Sensor surface spots immobilised with 50 µg mL^{-1} anti-cTnT 1C11 or 30 µg mL^{-1} control antibody. 100 ng mL^{-1} cTnT injected for 3 minute (direct binding), followed by 3 minute injection of detector antibody (1 – 5 µg mL^{-1} anti-cTnT 7G7), followed by regeneration. Flow rate 25 µL min^{-1}.

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The binding responses showed that although the differences in binding responses were small, the optimum anti-cTnT 7G7 antibody concentration had shifted slightly to 3 µg mL^{-1}, from its previous optimum value of 2 µg mL^{-1}. The amplification of the cTnT remained at around 3-fold. These results indicated that the additives were unlikely to be causing a problem with anti-cTnT 7G7 antibody binding, as the amplification factor did not appear to be affected. The slight increase in optimum concentration could have been due to steric effects. Now that there was less cTnT binding occurring, there was probably more space sterically for anti-cTnT 7G7 antibody binding to take place.

3.4.8 Conjugation of AuNPs with anti-cTnT detection antibodies

Using a sandwich assay format has shown (in section 2.4.7) that the response obtained for the binding of cTnT can be greatly amplified, by between 6 and 10-fold. This increase in sensitivity is closer to that required for an LOD which would detect clinically relevant concentrations of cTnT in serum samples. However, it is still insufficient, and the immunosensor will not be able to measure the lower concentrations needed to distinguish between healthy individuals, and those individuals with moderately raised levels of cTnT, which would enable earlier diagnosis of acute myocardial infarction, and prevent any unnecessary further damage of myocardial tissue.

Sensitivity for SPR analysis can be further improved by the use of additional amplification methods together with a sandwich assay approach. The use of AuNPs conjugated to antibodies to enhance the response has been widely used and reported (Mitchell and Lowe, 2009; Fernandez et al., 2012; Saha et al., 2012; Li et al., 2013).

AuNPs were conjugated to the detection antibody (anti-cTnT 7G7 antibody) as depicted in Figure 3.10, using the procedure described in section 3.4.8. The conjugation, is not a conventional conjugation reaction, but is actually a physical-adsorption. This method is used preferentially as a first option to actual covalent binding, as it preserves the conformation of the antibody, helping to maintain high efficiency. This initial procedure appeared to work for

the preparation of the first batch of antibody conjugated AuNPs, but subsequent batches failed the quality check when the flocculation test was used.
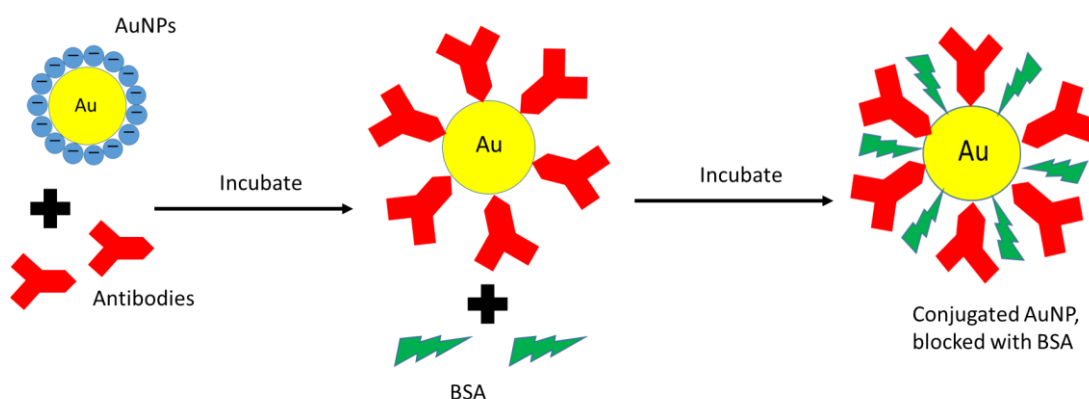
Figure 3.10:   Schematic of the antibody to AuNP conjugation process.

The flocculation test works on the principle that AuNPs will aggregate in the presence of high salt or ionic concentrations. When AuNPs are present in a colloidal solution they do not aggregate due to the repulsive forces from their surface charge, as the energy required to overcome this repulsion is too great. During the conjugation process the pH is increased to approximately pH 8.9 by the addition of the NaOH, which allows the antibody molecules to overcome the energy barrier and move closer to the AuNPs to interact with binding sites. Similarly, during the blocking procedure the BSA binds to any remained active binding sites on the AuNP.

When a concentrated solution of NaCl is added to the reconstituted conjugated AuNPs, if the AuNP's surface is fully bound with antibody and BSA, this will prevent the aggregation of the AuNPs. If the coverage is incomplete or non-existent, the NaCl ions shield the repulsive charges and reduce the energy barrier, thus allowing the particles to interact and aggregate, as shown in Figure 3.11. The colour change occurs because the aggregation of the AuNPs causes an electronic coupling interaction between the localised surface plasmons of the AuNPs. The surface plasmon resonance for small AuNPs leads to absorption

of blue-green light, leaving red light to be reflected. As the AuNPs aggregate and get larger, the wavelength of the surface plasmon resonance changes, shifting towards the red wavelengths, resulting in blue light being reflected (Ghosh and Pal, 2007).

Negative charges repel each other, and prevent aggregation

High NaCl concentrations shield the negative charges, allowing partially conjugated AuNPs and AuNPs to approach each other, and aggregate into larger clumps
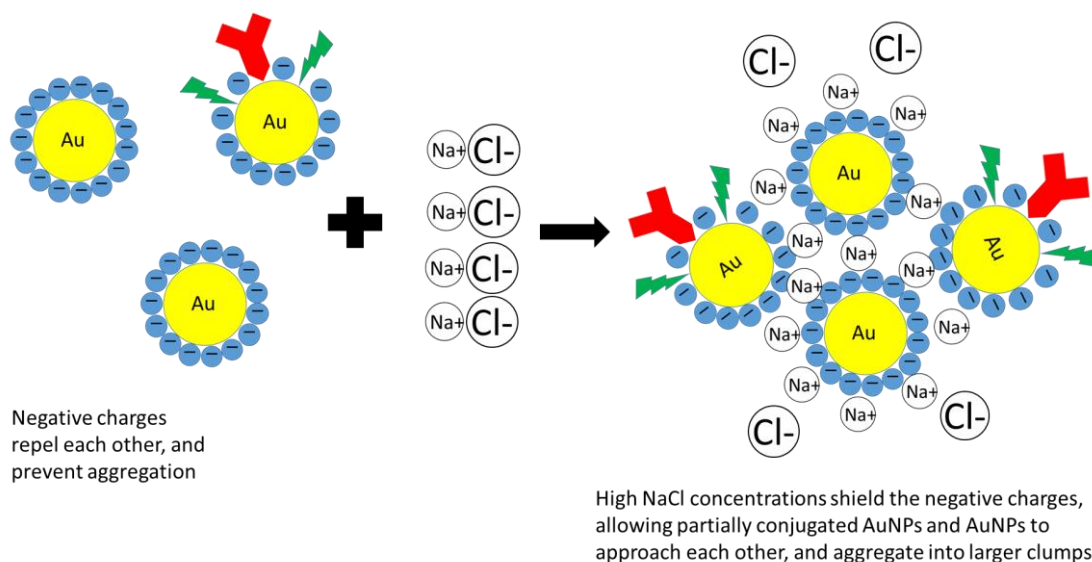
Figure 3.11: Schematic illustration of colloidal AuNPs and partially conjugated AuNPs in solution, and the aggregation that occurs on addition of 2.5M NaCl solution.

3.4.9 Optimisation of Au nanoparticle size

Different sizes of AuNP including 20, 40 and 60 nm were investigated to find the optimum size for the SPR affinity assay.

The visible results of the conjugation gave an early sign as to which particle size may be the most effective. The photograph in Figure 3.12 shows the different colours of the solutions of conjugated AuNPs, after they were tested for aggregation by adding 2.5M NaCl. The 20 nm size AuNPs instantly went a dark blue colour, indicating poor conjugation with lots of free gold nanoparticles aggregating together. Due to the smaller size of the nanoparticle, through steric effects, it is likely that insufficient antibodies can conjugate to it, leaving it open

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to aggregation. The 40 nm size AuNPs remained the original red colour, indicating good conjugation with the antibodies. Whilst the 60 nm nanoparticle size solution became a pale pink-purple colour, indicating that there was some conjugation to the antibodies and also some aggregation of unbound nanoparticles. The reason for this was not clear, but one possibility is that a greater excess of antibodies was required (because of the greater surface area of the particle) for the conjugation process to be efficient (Cytodiagnostics, 2014).
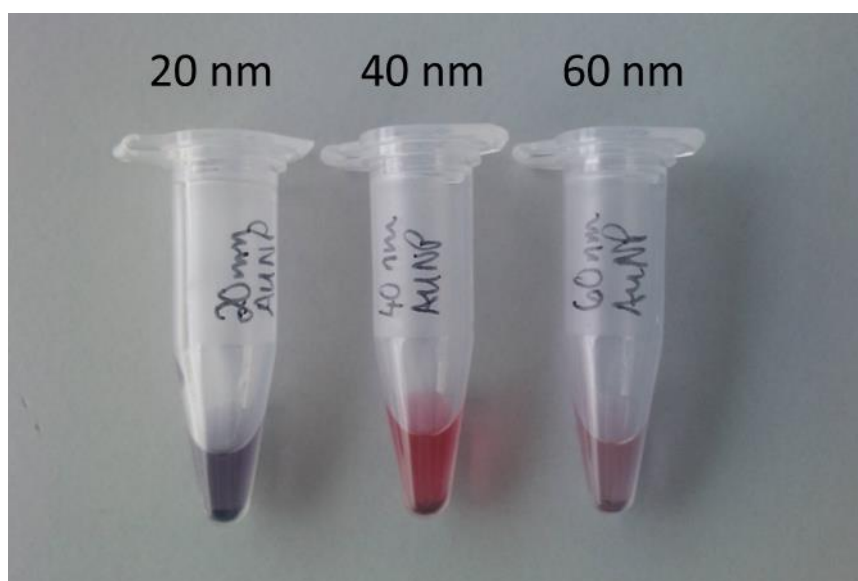
Figure 3.12: Photograph of solutions of different sized Au nanoparticles conjugated to cTnT antibodies.

The different sizes of conjugated AuNPs were tested for their ability to amplify the response as described in section 2.3.9, using the sandwich assay format. Different fixed concentrations of cTnT (25, 50 and 100 ng mL^{-1}) were used for this investigation, and the responses obtained for direct binding, prior to injection of detection antibody were recorded. The results are shown in Figure 3.13.
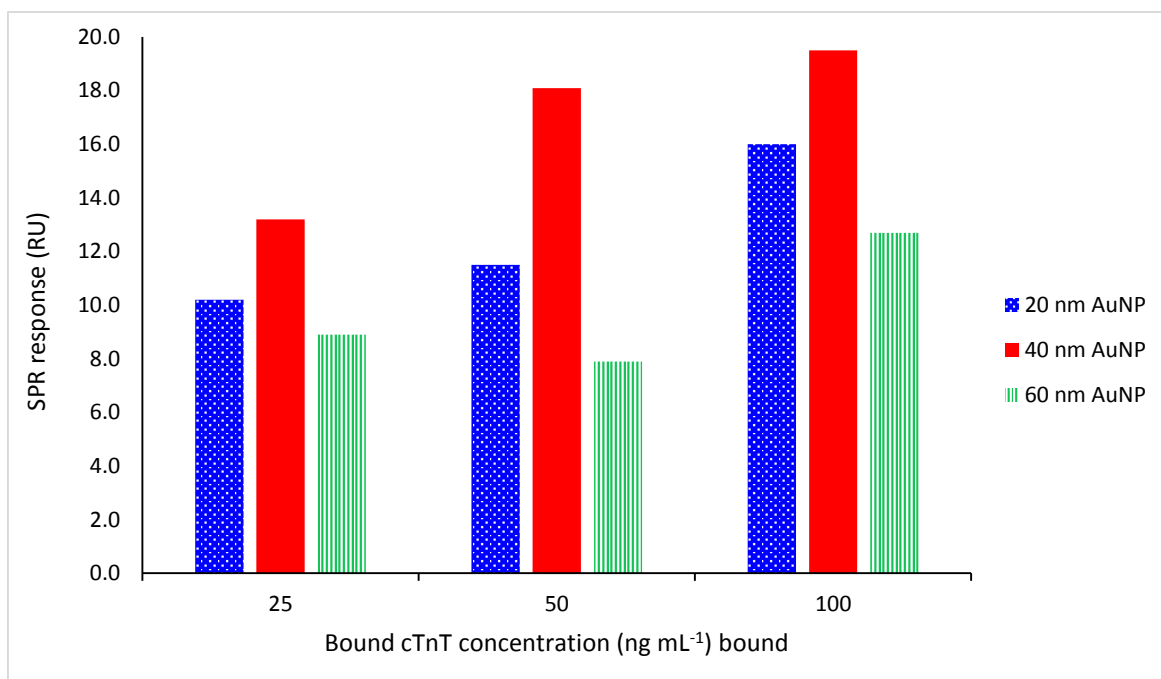
Figure 3.13: Comparison of binding responses amplified with antibody conjugated AuNPs at different bound cTnT concentrations. Sensor surface immobilised with 50 µg mL^{-1} anti-cTnT 1C11. 25, 50 or 100 ng mL^{-1} cTnT injected for 3 minute (direct binding), followed by 3 minute injection of AuNP conjugated detector antibody (20, 40 or 60 nm size), followed by regeneration. Flow rate 25 µL min^{-1}.

For the sandwich assay at each cTnT concentration, and each AuNP size, there was an amplification of the response obtained, although it was minimal for the conjugated 60 nm AuNPs. A larger increase in response was seen for the conjugated 20 nm AuNPs, but from the aggregation observed in solution, this is likely to have been mostly due to non-specific binding of the aggregated AuNPs. The biggest increase in SPR response was observed with the conjugated 40nm AuNPs, which gave a mean amplification of approximately 70% compared to the values obtained for the direct assay. Thus, 40 nm gold nanoparticles were chosen as the optimum size for the final assay method. This size correlates well with that found to be the optimum when conjugating antibodies to AuNPs for use in lateral flow immunoassays (Rivas et al., 2014).

3.4.10 Confirmation of the AuNP conjugation procedure

The amplification of binding response obtained when using the AuNP conjugated detector antibodies appeared to be much lower than expected (a maximum of 3-fold, rather than 10-fold). Thus, the protein content of the conjugated AuNPs was investigated to examine if the antibodies were actually being conjugated to the nanoparticles or not.

The method used to assess the protein content was the bicinchoninic acid (BCA) protein assay method (Smith et al., 1985). The Cu^{2+} ions from the copper II sulphate are reduced to Cu^+ ions when they react with the peptide bonds of a protein, whether from the antibody or the BSA. Each of the Cu^+ ions then chelates with two molecules of bicinchoninic acid, which causes a colour change from the blue-green of copper II sulphate to the purple chelated product. This colour change is measured colorimetrically at an absorption wavelength of 562 nm. The amount of Cu^+ produced is directly proportional to the amount of protein present.

An initial test run was conducted to establish the method. The results as presented in Figure 3.14 showed that the linearity and precision of the method was excellent (R^2 = 0.9961, with CV's across the calibration range of 0.1 to 2.4%).
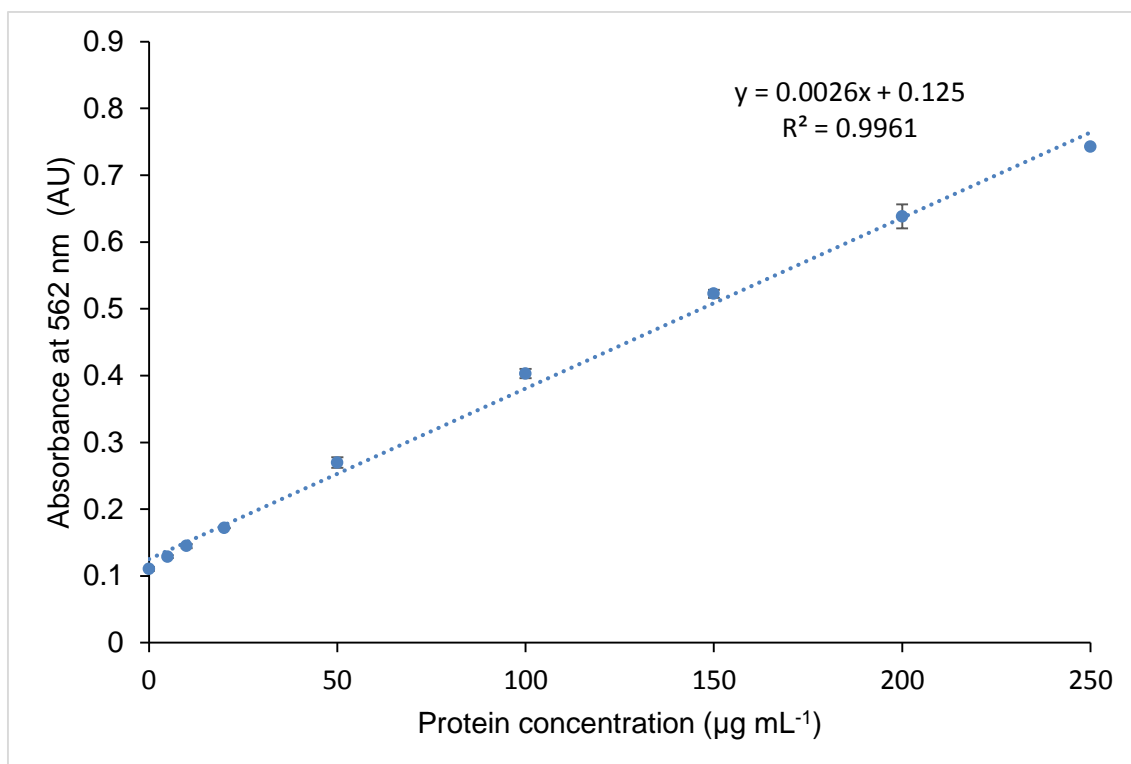
Figure 3.14: Plot of total protein concentration against spectrophotometric absorbance for BCA assay of protein content. Protein used for calibration was mouse IgG. Error bars represent SD, n=3.

Two identical samples were taken through the conjugation procedure, one was centrifuged and analysed after the incubation to conjugate the detector antibodies to the AuNPs prior to blocking. The other sample was allowed to continue through the procedure and be incubated with BSA for blocking, before centrifugation and analysis. Samples of either blank, control, calibration standard or unknown sample (i.e. supernatant or reconstituted samples from the conjugation procedure) were analysed in triplicate for protein content using the BCA method. The calibration standard sample results were used to construct the calibration curve, and the protein content of the unknown samples was determined from the calibration curve, using the following equation;

$$x = (y - c)/m \ \text{ (derived from } y = mx + c)$$

The percentage of the total protein content was calculated as the amount of protein measured compared to the amount of total protein expected if the conjugation had been 100% effective.

The results for the protein determination of the supernatant and reconstituted AuNP conjugated antibodies (Table 3.2) showed that only a very small amount of antibodies have been conjugated to the AuNPs (3%). Whilst some losses during the conjugation procedure are expected, this low percentage helps to explain the poor amplification observed when using these batches of conjugated AuNPs in the subsequent sandwich assay. If the percentage of antibodies conjugated to the AuNPs could be increased further, it is likely that the amplification of the binding response signal could be increased much higher, closer to the 10 to 32-fold amplification published for other assays (Li et al., 2009; Uludag and Tothill, 2010; Cao et al., 2011; Fernandez et al., 2012; Li et al., 2013).

Table 3.2: Percentage of total protein determined after conjugation of AuNPs with anti-cTnT 7G7 antibody before and after blocking with BSA. To conjugate, AuNPs were incubated with 20 µg mL^{-1} of anti-cTnT 7G7 antibody, for 1 hour. Samples taken through blocking procedure were incubated with 10 mg mL^{-1} for an additional 20 minutes.

Batch identity	% protein conjugated to AuNPs	
	Before blocking	After blocking with BSA
Early batch	-	97.5
Batch 1	6.0*	86.5
Batch 2	3.0	45.3

* Estimated by back-calculation

3.4.11 Detection of cTnT in 50% human serum

A sandwich assay (using anti-cTnT 7G7 antibody as the detector antibody) was run under the previously optimised conditions using a calibration curve prepared with cTnT spiked into 50% human serum, over the concentration range of 0.5 to 40 ng mL^{-1} cTnT. The difference in responses at the lowest concentrations of cTnT was only 1 to 2 RU (as shown in Figure 3.15, and more clearly in the

zoomed-in inset graph), which is too small to distinguish an LOD at those concentrations. This was caused by the non-specific binding taking place, giving a high background signal. The overall binding response was also reduced due to the non-specific binding additives used in the buffer for diluting the serum, the greatest effect coming from the increased NaCl concentration. Thus, the LOD for the regular sandwich assay was only about 8.64 ng mL^{-1} cTnT in human serum.
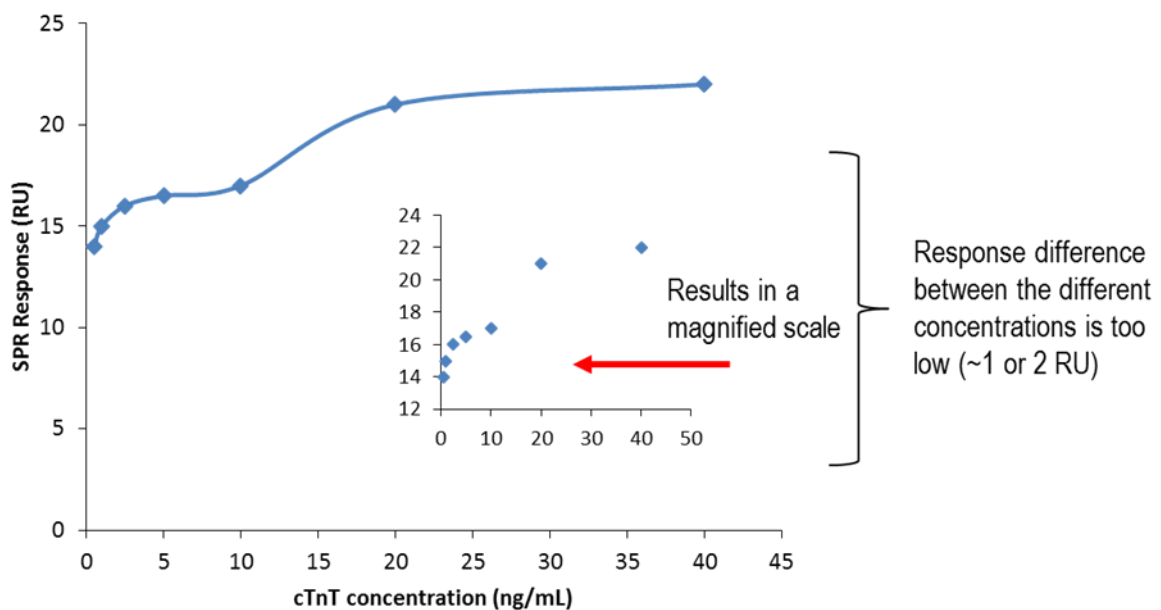
Figure 3.15: cTnT sandwich assay binding responses obtained for cTnT samples prepared in 50% human serum; with inset showing the detail of responses at the lowest concentrations on magnified scale. cTnT spiked into 50% human serum were injected onto 50 µg ml^{-1} anti-cTnT 1C11 (capture) antibody immobilised sensor surface. Later anti-cTnT 7G7 detector antibody was injected, and the binding responses recorded.

The sandwich assay was then repeated for comparison using 3 µg ml^{-1} anti-cTnT 7G7 antibody conjugated AuNPs to further amplify the signal, over the same concentration range (0.5 to 40 ng mL^{-1} cTnT). The calibration samples were again spiked into 50% human serum, and injected in triplicate. The sensorgram in Figure 3.16 illustrates that the NSB additives in the sample buffer with which the serum was diluted, were working effectively as there is no

difference in binding response after an injection of cTnT followed by the injection of the AuNP modified detector antibodies, on the control spot.
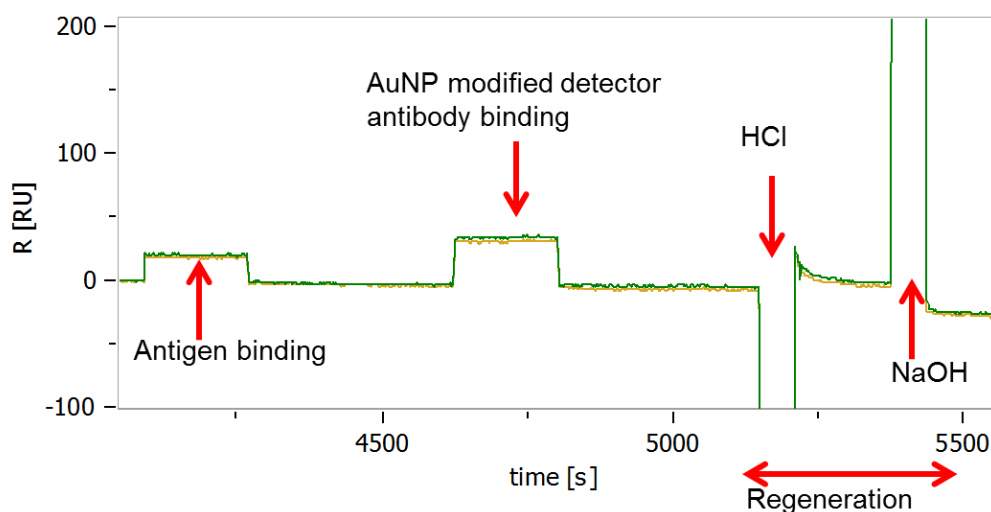
Figure 3.16: Real time sensorgram of binding assay using AuNP modified detector antibodies on control sensor surface to evaluate non-specific binding.

When the lowest concentration cTnT spiked serum (0.5 ng mL^{-1}) was injected, the net change in cTnT binding response was approximately 4 RU. The binding response was then amplified on the subsequent injection of the AuNP modified detector antibody by approximately three fold. The actual cTnT detection antibody binding responses were obtained after subtraction of the non-specific binding response obtained for the control antibody, and the binding responses achieved are shown in the calibration graph in Figure 3.17. The enhancement of the signal, meant that cTnT concentrations down to an LOD of 0.5 ng mL^{-1} of cTnT could now be detected. The difference in signal between the lowest concentrations was now increased to 10 RU; the larger difference was better as it improves the accuracy and robustness of the immunosensor. The LODs obtained for each stage of the immunosensor development are summarised in Table 3.3.
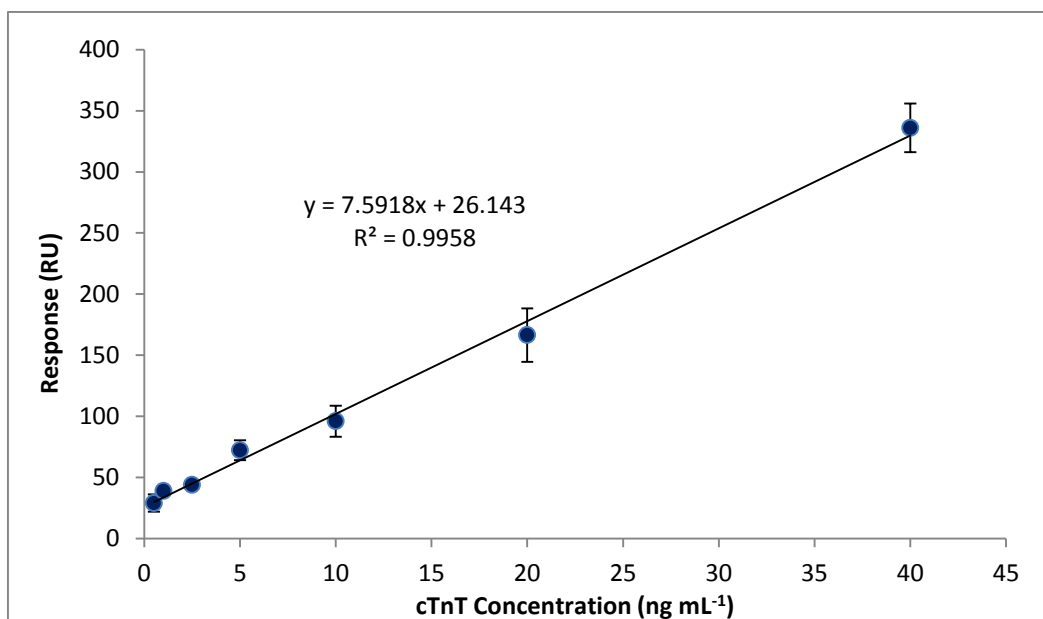
Figure 3.17: Calibration plot for the measurement of cTnT in human serum using detector antibody conjugated AuNPs for signal amplification of the binding response. cTnT spiked into 50% human serum was injected onto 50 μg ml^{-1} anti-cTnT 1C11 (capture) antibody immobilised sensor surface. Later anti-cTnT 7G7 detector antibody modified AuNPs were injected, the binding responses recorded and calibration curve obtained.

Table 3.3: Summary of cTnT assay results obtained using SPR detection

	Direct assay in PBS/T	Sandwich assay in PBS/T	Sandwich assay in 50% serum	Sandwich assay in 50% serum using AuNP modified antibodies
LOD	16.9 ng mL^{-1}	4.43 ng mL^{-1}	8.64 ng mL^{-1}	0.50 ng mL^{-1}
Linear range	25 – 400 ng mL^{-1}	5 – 200 ng mL^{-1}	5 – 150 ng mL^{-1}	0.5 - 40 ng mL^{-1}
Equation	y = 0.223x + 10.9 (R^2 = 0.991)	y = 0.632x + 21.3 (R^2 = 0.988)	y = 1.18x + 18.7 (R^2 = 0.995)	y = 7.59x + 26.1 (R^2 = 0.996)

All calibration standard samples prepared using PBST sample buffer with added NSB additives

3.5 Conclusion

This chapter describes the second phase in the development of a sensitive immunosensor for the determination of the cardiac biomarker cTnT in human serum using SPR optical based detection. The second development phase focussed on two different issues. The first one was the investigation of the non-specific binding of cTnT itself, progressing onto the non-specific binding of serum matrix components such as other proteins. The aim was to reduce non-specific binding as much as possible, and incorporate a control antibody within the immunosensor to monitor and compensate for any remaining non-specific binding. The second issue was the investigation of using gold nanoparticles for signal amplification in the sandwich immunoassay, by conjugating them to the detector antibody.

The initial experiments to set up a control antibody as one of the four sensor surface spots on the sensor chip, identified that there was a large amount non-specific binding of the cTnT to the sensor chip; even after using a SAM on the sensor surface and blocking with two different molecules (BSA and ethanolamine) following the immobilisation of the capture antibodies. The non-specific binding was investigated in more detail, but no specific location was identified and it appeared the cTnT was binding to all surfaces. More detailed analysis of the sensor surface would be required using atomic force microscopy (AFM), to visualise if there was non-uniformity or defects on the sensor surface. This would help to identify if there were any defects on the sensor surface, which might be creating sites to which non-specific binding may occur, as illustrated in Figure 3.18.
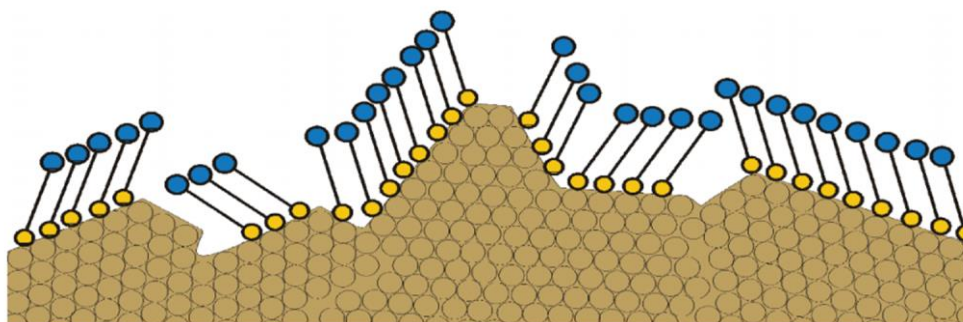
Figure 3.18: Schematic of possible defects of SAMs adsorbed onto a gold surface on a molecular scale, where non-specific binding interactions may occur.

(reproduced from E.T. Getig, Chapter 6, Surface Chemistry in SPR technology, in "Handbook of Surface Plasmon Resonance", (2008), eds. Schasfoort RBM. and Tudos AJ., RSC Publishing).

Using AFM, Uludag (2010) found after three re-uses of a similar gold coated QCM sensor chip, small white dots were observed on the gold surface (oxygen plasma was used for cleaning the sensor chips). This could indicate that even after cleaning the SPR sensor chips with piranha solution, microscopic impurities may have still been present, which would lead to the formation of an incomplete SAM. Small defects in the SAM, would then provide sites for non-specific binding to occur, although this should have been minimised by blocking with the ethanolamine. Over the course of this project, some of the chips used, would have been re-used more than three times, so if these defects were present they may have contributed to some of the variable results and non-specific binding observed.

The blocking process was examined to evaluate if any non-specific binding related to incomplete blocking could be reduced. Different concentrations of the BSA solution used for blocking were tested, as were multiple injections of BSA and ethanolamine, and blocking with skimmed milk proteins was also tested. However, none of these appeared to have any effect on reducing the non-specific binding of cTnT, suggesting that the original blocking process was blocking all the available sites to which the reagents had access.

After the optimisation of the blocking procedure, the use of additives to reduce the non-specific binding was investigated. Three different additives were tested; BSA, NaCl and dextran. The NaCl was found to be the most effective at reducing the non-specific binding of cTnT, whilst the other two had a negligible effect. Dextran has been shown to help reduce non-specific binding when it is used with sensor chips coated with carboxymethyl dextran hydrogel (Situ et al., 2008).

The use of NaCl in the sample buffer was found to eliminate all the non-specific binding of cTnT to the control antibody, although it did also reduce the cTnT binding response overall as well, thus potentially affecting the sensitivity of the immunosensor. The use of the optimised sample buffer (with NaCl and BSA additives) was tested with human serum samples, to evaluate its effect on the non-specific binding of serum matrix components. The additives were found to reduce the non-specific binding by 93% and 73%, when using a 10% or 50% solution of serum, respectively, but could not eliminate it entirely. A choice was made to go forward using the 50% diluted serum, as this would give the best compromise between reducing non-specific binding, whilst trying to maintain as much cTnT binding response as possible. To calculate the actual binding response due to the cTnT itself, the binding response measured on the control antibody sensor spot would be subtracted from the response measured for cTnT binding on the anti-cTnT antibody sensor spots.

The last part of the development carried out was to investigate if the response obtained for the cTnT binding could be enhanced any further, beyond that obtained by using a sandwich immunoassay format. The method chosen for amplification of the signal was to conjugate AuNPs to the detector antibody, as this had been successfully employed for amplification in many other immunosensors (Uludag and Tothill, 2010; Bedford et al., 2012, Fernandez et al., 2012; Li et al., 2013).

The optimum size of AuNP for signal amplification was assessed to be 40 nm. There were some difficulties in achieving maximum conjugation of the antibodies to the surface of the AuNPs. In theory, a maximum of 150 to 210

antibodies should be able to conjugate to each 40 nm size gold nanoparticle (Amsbio, 2014; Tedpalla, 2014). However, the quality checks with 2.5M NaCl and the total protein results from the BCA assays showed, the level of conjugation achieved was quite poor, as only approximately 3 to 6% of the antibodies were conjugated.

The effect of using of the AuNP conjugated detector antibodies on signal enhancement in the sandwich immunoassay, instead of unmodified detector antibodies, was evaluated and compared. The samples used for this were cTnT calibration samples which had been prepared in human serum which had been diluted 50% with PBS/T buffer containing NSB additives. The results showed that in serum, the amplification achieved with unmodified detector antibodies was not as good as that previously found in samples prepared in buffer. The amplification obtained with the AuNP conjugated nanoparticles was much better, especially once the non-specific binding (as assessed by the control antibody) had been subtracted. There was a 10 RU difference in cTnT binding response between the two lowest calibration concentrations (0.5 and 1 ng mL^{-1}), whereas with unmodified detector antibodies, this difference was only 1 to 2 RU.

The developed immunosensor was evaluated over a concentration range of 0.5 to 40 ng mL^{-1} cTnT, and the LOD estimated to be 0.5 ng mL^{-1} cTnT. This was a great improvement on the LODs obtained for the direct capture assay and the unmodified detector antibody sandwich assay, which were 16.9 and 4.59 ng mL^{-1} cTnT, respectively. An immunosensor with an LOD of 0.5 ng mL^{-1} cTnT could only be used to diagnose a major heart attack after cTnT concentrations had reached their maximum serum concentrations (i.e. 1 ng mL^{-1} cTnT), (Twerenbold et al., 2012). However, to meet the ultimate aim of developing a highly sensitive immunosensor for detection of cTnT at concentration levels which could allow early diagnosis of a minor heart attack and distinguish these concentrations from those obtained in normal healthy individuals, an LOD of at least 0.05 ng mL^{-1} is needed. Therefore, further development of this immunosensor would be required to achieve the requisite sensitivity.

CHAPTER 4

FINAL DISCUSSION AND CONCLUSION

4 Final Discussion and Conclusion

4.1 Overview

This thesis describes the development of an immunosensor for the determination of the cardiac biomarker Troponin T in human serum using SPR based optical detection. The initial development of the immunosensor was performed using solutions of cTnT in buffer. A number of different basic aspects of the immunosensor were investigated; such as SAM deposition, optimum concentrations of both capture and detection antibodies, immobilisation pH and running buffer. The optimised conditions were used to assess the LOD that was achievable when the immunosensor was used in both direct and sandwich immunoassay formats. The second stage of the development focussed on improving the LOD of the immunosensor in human serum. This involved examining the extent of non-specific binding and investigating how best to minimise it, whilst also investigating the amplification of the signal through the use of detector antibodies conjugated to AuNPs. The optimised conditions were implemented and used to assess the sensitivity of the developed immunosensor for the determination of cTnT in human serum.

4.2 Immunosensor development

Ensuring that the sensor surface is covered completely and uniformly with a SAM is the foundation to developing a good immunosensor. The quality of the SAM impacts on many aspects of the subsequent preparation, use and stability of the immunosensor, whether it is used for a direct assay or a sandwich assay. Any defects in the SAM deposition, no matter how "nanoscopic" will cause problems. Exposed active sites on the gold surface can easily bind to many different molecules especially proteins. This may cause antibodies subsequently bound to the sensor surface, to lose their binding affinity by denaturing the 3-D structure of the antibody, or encourage poor orientation of the antibodies, so that their F_{ab} binding sites are less available for binding to the antigen. Also if the SAM deposition is poor, it will affect the maximum density of capture antibodies which can be immobilised onto the sensor surface. All these

three outcomes will lead to a reduction of the maximum amount of cTnT which can bind to the immunosensor, and so reduce the immunosensor's sensitivity. In this project the consistent results obtained for capture antibody immobilisation binding response (when using freshly piranha-cleaned chips and commercially prepared chips) suggests that close to the maximum amount of antibody possible, was being immobilised.

The other issue that arises when a SAM has nanoscopic defects like pinholes, is that non-specific binding will increase, again negatively impacting on the realisable sensitivity of the immunosensor. This was found to be a major issue during the development of this cTnT immunosensor, as the non-specific binding of both cTnT and serum proteins was found to be very high. The non-specific binding of cTnT itself was eliminated by the addition of NaCl to the sample preparation buffer. Non-specific binding of serum proteins could not be eliminated entirely, but was reduced to more manageable levels through the addition of NaCl and BSA to the sample preparation buffer. However, the addition of NaCl did adversely affect the overall binding responses obtained, and so ultimately the sensitivity of the immunosensor.

There was a substantial amount of non-specific binding of serum proteins observed, even when BSA or milk proteins and ethanolamine were used to block any active sites after immobilisation. Therefore another approach worth investigating, could have been blocking with the serum proteins themselves, by injecting serum onto the sensor surface (Masson et al., 2007). The serum used would have to be blank serum from a non-mammalian species (such as chicken or fish) to minimise the potential for cross-reactivity (Garay et al., 2010; Biocompare, 2012). Using serum could minimise the background signal from serum protein binding, and thus allow a clearer differential of the signal due to cTnT binding response, so increasing sensitivity. Blocking with serum protein may also allow the use of more concentrated or even undiluted serum samples, giving another opportunity to improve the LOD of the immunosensor.

If the non-specific binding to the SAM in the first instance could be minimised further, then this could allow a lower concentration of NaCl to be used in the

sample buffer to eliminate any remaining non-specific binding of cTnT. Lowering the NaCl concentration would increase the binding response for the cTnT binding, and so improve the LOD attainable and the sensitivity of the immunosensor.

11-MUDA was chosen as the alkanethiol in the preparation of the SAM for this immunosensor as many groups had used it successfully (Ayela et al., 2007; Altintas et al., 2011; Stan et al., 2012), and it was the SAM used for the Sierra Sensors commercial standard amine chip. To improve the effectiveness of the SAM for reducing non-specific binding for SPR-based immunosensors it may be worth exploring applying a mixed SAM, as many groups have used different combinations to successfully reduce non-specific binding. Lee et al., (2005) and Stan et al., (2012) both successfully used 11-MUDA and 3-mercaptoproprionic acid, a 12-MUDA and 1-heptanethiol combination was used by Yoon et al., (2011), and Li et al., (2013) used 11-MUDA with 16-mercaptohexanoic acid. It is thought that applying a SAM consisting of molecules of two different chain lengths gives better coverage of the sensor surface with fewer pinhole defects, whilst also increasing the surface area available for immobilisation of ligands, thus reducing non-specific binding and increasing sensitivity at the same time.

Apart from changing the molecules used to form the SAM, there is also the potential to reduce the non-specific binding by modifying the SAM surface. Ayela et al., in 2007 used 11-mercapto-undecane derivatives with polyethylene glycol (6 ethylene glycol units) to form a mixed SAM with 11-MUDA, which reduced the non-specific binding by 8-fold. Similarly, oligoethylene glycol terminated alkanethiols used to form SAMs have been shown to be effective at reducing fouling by serum proteins (Cao et al., 2006). Thus it may be worth investigating using glycol derivatives of alkanethiol molecules with standard alkanethiols in a mixed SAM with in the further development of this immunosensor.

Experiments were then conducted to optimise the immobilisation conditions for attaching the capture antibody: examining ligand concentration, pH of

immobilisation and immobilisation time. The results from these experiments showed that increasing the ligand concentration, reducing the pH and increasing the immobilisation time, all improved the cTnT binding response. A decision was made to use a 50 µg mL^{-1} concentration of anti-cTnT 1C11 antibody for immobilisation, and a 3 minute immobilisation time, as the gains in extra cTnT binding response going beyond these amounts, was not proportional to the extra anti-cTnT 1C11 antibody used. The addition of the detergent Tween 20 to the running buffer was also found to increase the cTnT response. The detergent reduces the non-specific binding by disrupting the ionic and hydrophobic biomolecule to sensor surface bonds.

Once the initial optimum conditions were established, the immunosensor was assessed using a direct assay format, measuring the binding response of the cTnT to the capture antibody, using calibration standard samples prepared in PBS/T buffer. The calibration was found to be linear, and the LOD was determined as 16.9 ng mL^{-1} cTnT. Whilst this was a good LOD for a "label-free" immunosensor, and could be a viable LOD for some cardiac biomarkers (e.g. C-reactive protein or myoglobin), it was not sensitive enough to measure cTnT in clinical samples.

A sandwich assay format was then utilised, to enhance the signal and improve the LOD. A second anti-cTnT antibody (anti-cTnT 7G7 – which binds to a different epitope region) was used as the detection antibody for the signal enhancement. Once the concentration of the detection antibody to be used was optimised, the sandwich assay was assessed as previously done for the direct format assay. The LOD of the sandwich format immunosensor was established at 4.59 ng ml^{-1} cTnT in PBS/T buffer. This was approximately 4-fold more sensitive than for the direct assay, and better than the 100 ng ml^{-1} cTnT achieved by Lui et al., (2011), who also developed a cTnT SPR-based immunosensor. However, even greater sensitivity was required to be able to measure down to the concentrations needed (< 0.06 ng ml^{-1} cTnT) for diagnosis of myocardial infarction (Jaffe et al., 2012).

To further increase the sensitivity of the immunosensor, signal amplification using AuNPs conjugated to the detector antibodies was studied. Preliminary experiments in PBS/T buffer suggested that the signal could be amplified by at least 10 to 32-fold, which was in line with the results obtained by other groups (Uludag and Tothill, 2010; Li et al., 2013). When the AuNP modified detector antibodies were initially tested in the sandwich assay using samples prepared in serum, the amplification of signal was not as high as expected. This was partly due to the non-specific binding issues with serum proteins as described earlier, and having to use NSB additives in the sample buffer. However, there also appeared to be an issue with the AuNP conjugation process itself, which was examined in more detail. Investigations measuring total protein content (with and without blocking) showed that the percentage of antibodies being conjugated to the nanoparticles was only approximately 3 to 6%, which may explain the lower amplification.

At the end of the project, the sandwich assay method developed so far (using optimised conditions) was assessed by preparing calibration standard samples in 50% serum, diluted with PBS/T buffer with NSB additives. The results showed that an LOD of 8.64 ng mL^{-1} cTnT was achieved when using unmodified detector antibodies in the sandwich assay, whilst a much more sensitive LOD of 0.5 ng mL^{-1} cTnT was achieved using AuNP modified detector antibodies.

The LOD of 0.5 ng mL^{-1} cTnT achieved by the immunosensor developed in this project, is very close to the criterion level of 0.3 ng mL^{-1} cTnT advised by WHO for diagnosis of an MI using the cTnT cardiac biomarker (Allender, et al., 2008). Only minor refinement of the developed cTnT immunosensor would be required to reach this criterion level. To distinguish healthy individuals from those in the very early stages of a MI (less than 3 to 4 hours post MI) before cTnT levels have had an opportunity to build up in the bloodstream; the cut off level of cTnT is suggested by WHO is 0.01ng ml^{-1}, whilst Jaffe et al., (2012) state 0.06 ng ml^{-1}. Thus, to reach the ultimate goal of a highly sensitive immunosensor (which could distinguish between healthy individuals and those

with slightly raised cTnT levels), additional development would be required, to further reduce the non-specific binding, and improve the percentage of antibodies conjugated to the AuNPs, increasing amplification.

The cTnT immunosensor developed has some additional advantages compared with the other cTnT immunosensors cited in Table 1.3. The procedure for immobilising the antibodies on the sensor surface, at just under 30 minutes, is the fast, and combined with sample analysis time, is only 55 minutes. Whereas with the other cTnT immunosensors total immobilisation plus analysis time varies from 45 minutes to 3 days (de Avila et al., 2013). This lends itself to the development of a re-usable immunosensor, which can measure patient samples in "real time". The format of this immunosensor is a straightforward sandwich assay performed on a simple sensor chip, which could easily be "miniaturised" and used in a small portable SPR device, which can be taken out into the field and used in remote locations. This will become of increasing importance as the rates of CVD start rising much faster in developing countries over the coming decades.

4.3 Final conclusion

The overall results from this project include the development of an immunosensor for the cardiac biomarker cTnT using different assay formats, and testing it with spiked human serum samples.

The key achievements are listed below;

- Developing an immunoassay for cTnT using SPR for optical based detection
- Elimination of non-specific binding of cTnT, and reduction of serum protein binding by 93% in 10% serum and 73% in 50% serum.
- Enhancing the sensitivity of the assay with the use of AuNP conjugated detector antibodies.
- Optimisation of the cTnT immunosensor performance, to give the LODs as summarised in Table 3.3.

4.4 Future work

There are several directions which future research could take to improve the sensitivity of the cTnT immunosensor, to help distinguish between healthy individuals and those with slightly raised cTnT levels due to early or minor myocardial damage, if the funding was made available.

Reducing non-specific binding of cTnT and serum proteins further would improve the sensitivity of the assay, both directly by removing background noise, and indirectly by allowing the NaCl content to be reduced. This could be tackled by investigating the use of mixed chain length alkanethiols and/or ethylene glycol units (PEG) for the SAM, to better prevent non-specific binding to the sensor surface. The blocking procedure could be improved further by applying blank serum (from a non-related species such as chicken or fish serum, to minimise cross-reactivity), after immobilisation or prior to analysis as a pre-treatment.

Another route to increasing sensitivity further, would be more signal amplification. Currently the percentage of detector antibodies conjugated to the AuNPs is very low, thus if this could be increased, the signal would be enhanced. Suggestions for improving this, would be more optimisation of the conjugation conditions (e.g. examining different buffers, pHs, concentrations of reagents, and alternative blocking reagents). A heterobifunctional PEG linker molecule could be used during the conjugation procedure, which would help with the attachment of multiple antibodies in the correct orientation, and also stabilise the AuNPs, improving resistance to aggregation.

To complete the project, the final immunosensor would need to be tested using real patient samples. A cross-platform study would need to be conducted, where the same patient samples are analysed on the developed immunosensor and also on a standard approved method (e.g. a chemiluminescence immunoassay run on a hospital autoanalyser) in parallel for comparison. This would evaluate how close the agreement is between the results obtained by the two different techniques, and how well the developed immunosensor performs.

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APPENDICES

Appendix A   Publications

Conference Poster

M. PAWULA, Z. ALTINTAS, I.E. TOTHILL.
A sensitive and rapid determination of Cardiac Biomarker Troponin T using an Optical
Biosensor. *Poster presented at: BIOMARKERS - from Discovery to Clinical Diagnostics;
Cambridge, UK.* 8-9 July 2014.

A Sensitive and Rapid Determination of Cardiac Marker Troponin T using an Optical Biosensor

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Introduction

Cardiac troponin T (cTnT) is a cardio-specific, highly sensitive biomarker which is released immediately into the bloodstream following any myocardial damage such as acute myocardial infarction (AMI). Accurate, sensitive and rapid determination of cTnT allows early diagnosis of myocardial damage. This is very important for successful treatment of cardiovascular disease due to the faster diagnosis and initiation of the correct treatment which improves the prognosis for patients [1,2].

Methods/Materials

SPR sensors were incubated with a thiol solution to form self-assembled monolayer (SAM) on the gold surface prior to the immobilisation of anti-cTnT (1C11) capture antibodies via covalent binding. In a homogeneous bulk solution, cTnT was captured by anti-cTnT immobilised antibodies. Both direct and sandwich assay (using a second anti-cTnT (7G7) antibody for detection) formats were investigated and optimised. The response obtained was enhanced further by the use of gold nanoparticles (AuNPs) conjugated to the anti-cTnT detection antibody.
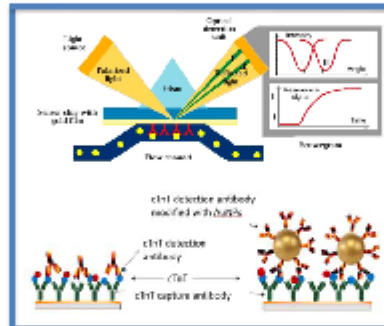

Fig 1. Schematic of cardiomyocyte cell and release of troponins into bloodstream.

Fig 2. Schematics of SPR detection and sandwich assays on sensor surface (with and without AuNPs).

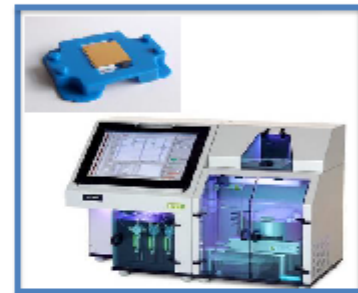Fig 3. An SPR sensor and the optical-based SPR-4 sensor system for cTnT determination (Sierra Sensors, GmbH).

Results

A variety of parameters were investigated to optimise immunosensor conditions; concentration of anti-cTnT capture antibody, pH of immobilisation buffer, running buffer and concentration of detection antibody. A direct binding assay was then successfully developed and the results were improved with sandwich assay using a detector antibody.

A regeneration method was developed using hydrochloric acid and sodium hydroxide, to enable reuse of the sensor multiple times. The SPR immunosensor showed good reproducibility and reliability for cTnT detection in the concentration range of 25-1000 ng mL^{-1} and 5-400 ng mL^{-1} for direct and sandwich assays, respectively. The linear regression analysis was performed and R^2 value was found to be 0.99 for both assays. Kinetic data analysis was applied through the SPR-4 analyser software to determine the affinity between anti-cTnT antibodies and the cTnT. The dissociation constant was found as 3.28 x 10^{-9}M using Langmuir binding model which indicates high affinity between cTnT and its target antibody.
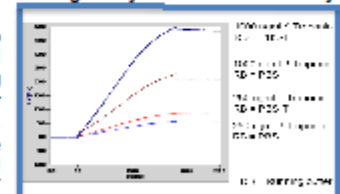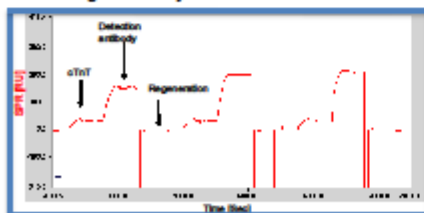

Fig 4. Optimisation of running buffer for cTnT detection

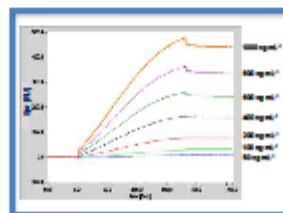Fig 5. Sensorgram of cTnT binding, followed by detection antibody injection and regeneration.

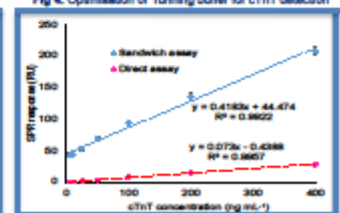Fig 6. Concentration dependent sensorgram of sandwich binding assay.

Fig 7. Comparison of direct and sandwich binding assays with standard deviations.

Conclusions

The proposed SPR immunosensor has promising potential to be developed for point-of-care testing for the early diagnosis of AMI. This method can also be used for the rapid detection of biomarkers in central nervous system diseases.

1.Jaffe, A. S. (2012), "Troponin—Past, Present, and Future", Current problems in cardiology, vol. 37, no. 6, pp. 209-228
2. Jin, J. -. and Chong, S. M. (2010), "Localization of the two tropomyosin-binding sites of troponin T", Archives of Biochemistry and Biophysics, vol. 500, no. 2, pp. 144-150.