DEVELOPMENT OF A MULTIPLEXING BIOSENSOR PLATFORM USING SERS PARTICLE IMMUNOASSAY TECHNOLOGY

by

NEELAM KUMARSWAMI

A thesis submitted to the University of Bedfordshire in partial fulfilment of the requirements of the degree of Doctor of Philosophy

March, 2014

DEVELOPMENT OF A MULTIPLEXING BIOSENSOR PLATFORM USING SERS PARTICLE IMMUNOASSAY TECHNOLOGY

NEELAM KUMARSWAMI

ABSTRACT

The purpose of this study is to demonstrate the ability of surface enhanced Raman scattering (SERS) active particles to enable multiplexed immunoassays in a lateral flow format for point of care (POC) testing. The SERS particles used for this study are chemically active glass coated gold particles, containing tracer molecules which in principle can be chosen to provide Raman Spectra with unique features allowing multiple tracers to be simultaneously measured and distinguished without interference between each other.

Lateral flow immunoassay technology is the important part of this study and can be conveniently packaged for the use of other than highly skilled technicians outside of the laboratory. A well-known (single channel - simplex) device for the pregnancy test is a typical example of the lateral flow assay. Similar formats have been/are being developed by others for a range of POC applications – but most diagnostic applications require simultaneous determination of a range of biomarkers and multiplexed assays are difficult to achieve without significant interference between the individual assays. This is where SERS particles may provide some advantages over existing techniques.

Cardiac markers are the growing market for point of care technology therefore biomarkers of cardiac injury (Troponin, myoglobin and CRP) have been chosen as a model. The object of the study is to establish the proof of concept multiplexing assay using these chosen biomarkers. Thus, initially all different particles were characterised in single and mixture

form. Also development of conjugate chemistry between antibodies for each analyte that have been purchased from commercial sources and SERS particles were analysed using different conditions like buffer, pH and antibody loading concentration to get the optimum intensity. The selected SERS particles and their conjugates were tested for size, aggregation and immune quality using a range of techniques: ultraviolet-visible (UV/Vis) absorption spectroscopy, dynamic light scattering (DLS) and lateral flow assay. These characterisations methodologies gave the understanding of optimum conditions of the each conjugates and individual's behaviour in mixture conditions as well.

After the characterisation all conjugates were tested singularly on the lateral flow assay using buffers and serum. The results of this single analyte immunoassay explained the individual's bioactivity on the lateral flow strip. Further in study, multiplex assay have been demonstrated in serum. These outcomes have described each candidate characteristic in a mixture form on the lateral flow strip. In order to get the optimum Raman intensity from multiplex assay, the detection and capture antibodies loading concentrations were tuned in the assay.

Later on different combinations (high, medium and low concentrations) of all three analytes were analysed and has found some interferences in multiplex assay. To investigate these issues various aspect were considered. First of all, different possibilities of non-specific interactions between the co-analytes and antibodies were tested. In addition, steric hindrance and optical interference investigations were performed via several assays and analysis using Scanning electron microscopy. The outcomes have confirmed related optical interferences. Therefore other assay (wound biomarkers) established to eliminate the interferences.

In summary, the works reported here have built and test the equipment and necessary reagents for individual assays before moving on the more complicated task. In addition, the entire study has given a deep knowledge of multiplex assay on a single test line including the investigation of the issues for selected cardiac biomarkers and their applications in the future.

ACKNOWLEDGEMENTS

First of all I would like to sincerely thank my husband for his understanding, patience, and most importantly, his support for my study. His encouragement was paramount for my long-term career goals. I would like to thank you Mr. Parthiv for giving unwavering love in past ten years and everything you have given me in this life. I dedicate this work to him.

I am deeply grateful to my supervisor, Dr Barry Haggett and Dr Robert Porter for their support with constructive comments for this study.

I would like to thank my parents who made me capable to tackle challenges in my life and making ambitious.

I am also thankful to National Physical Laboratory for funding this study.

Finally, and most importantly, I would like to thank my son who had been my strength for the study. He has been reducing my stress all the time by his priceless smile and affection.

DECLARATION

I declare that this thesis is my own work. It is being submitted for the Degree of Philosophy at the University of Bedfordshire.

It has not been submitted for any degree or examination in any other University.

Neelam Kumarswami **Signature: _____**

Date:

LIST OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1: Introduction

Chapter 2: Materials and Methods

Chapter 3: Calibration and characterisation

Chapter 5: Investigation of interference issues

<u>Chapter 6:</u> General discussion

CHAPTER 1: Introduction

1.0 Background to point of care

Point of care technology is often referred to as a quick, inexpensive, bedside detection technique for this there is a rapidly growing international market. POC technology is accomplished by the use of transportable, portable, and handheld instruments (*e.g.* homocystein and HIV tests, nerve conduction study device and blood glucose meter) Point of care testing has become established worldwide and finds a fundamental role in a public health (Tran and Kost, 2006). For early detection of human disease, biosensors form a major technology platform among POC techniques. Any biosensors that are based on immunoassay are presently extensively used in a range of areas, like research and development, clinical trials, diagnostics.(John *et al.,* 2008).

Lateral flow assay is the most extensively used technique to develop immunoassay biosensor devices. Currently most biosensor devices available on the market detect only a single analyte and employ optical or fluorescence-based detection that can be adversely affected by certain sample matrices, but there is a growing demand for biosensors to detect multiple analytes in complex mixtures (Brown and Doorn, 2008).

Multiplexing in a biosensor platform represents a target for diagnosis testing: the ability to screen for more than one analyte at the same time, can lead to more precise diagnosis because the variation in the amount of few disease related analytes gives a more specific signature over the variation of a single marker. Multiplexing assays can also be applied in the post-diagnosis stage, where it can be valuable in following the patient to ensure therapy effectiveness (Collinson, 2000).

However, to detect multiple analytes requires one or more separation steps and/or multiple methods of detection to avoid signal interference or quenching. Various platforms have been developed for multiplexing detection. For example fluorescence-based systems, surface plasmon resonance (SPR) and gold Nano rods based system (Huang *et al.,* 2012; Worsley *et al.,* 2012; Wu *et al.,* 2012)

Surface enhanced Raman spectroscopy (SERS) is another approach for sensitive multiplexed detection from biological samples and of growing interest (Brown and Doorn, 2008; Wang *et al.,* 2009) since it has the potential to achieve multiple analyte detection in one step reaction.

1.1 Immunoassay

Immunoassay is the most routinely used POC technique in many labs. ((a) Davies, 2005) In 1977 Berson and Yellow was awarded the Noble prize in physiology and medicine for developing the first radioimmunoassay. This was the first time ever that an immunoassay was reported (Wu, 2006). An immunoassay system requires two key elements:

1. The antibody raised against an analyte and
2. A labeled analyte –analogue.

The Principles of Immunoassay

An immunoassay is a biochemical test that measures the presence of a given analyte in a sample. In the core of any immunoassay is the reaction of antigen (analyte) with the specific antibody, upon which an immunocomplex is formed (Figure 1.1). Either antibody or antigen has to be labeled so that a measurable signal can be generated that corresponds to analyte concentration. The label is responsible for the high sensitivity of the assay, while the

selectivity is achieved by the fact that antibodies possess high specificity and affinity for their antigens ((b) Davies, 2005)

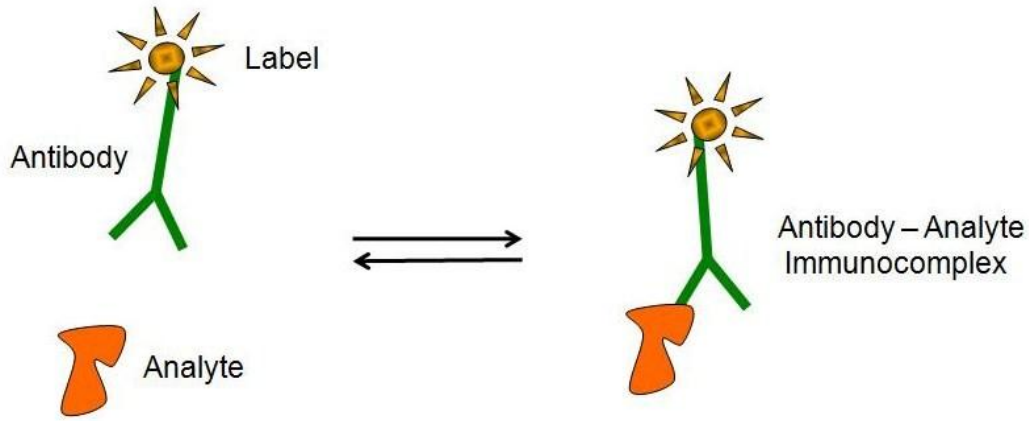


Figure 1.1: Formation of an immunocomplex – the core of immunoassay.

Different formats of immunoassay are based on the antigen used in the system. Competitive or non-competitive assay /and homogeneous or heterogeneous assay. In the competitive immunoassay the unknown concentration of unlabeled analyte is mixed with a known amount of labeled analyte. The competition for the antibody binding site is initiated. The labeled analyte will bind to the antibody therefore the output signal is inversely proportional to analyte concentration ((b) Davies, 2005). In a non-competitive immunoassay this involves two antibodies, one on a solid phase (capture antibody) and second on a labeled reagent in solution (detection antibody). The analyte is bound between two highly specific antibodies and so this system referred as a 'sandwich assay'. In this format with a higher concentration of the analyte, more labeled antibody will bind and so a greater signal will be produced.

In heterogeneous assays, the portion of antigen bound with the antibody is disconnected from the remaining unbound part after the reaction takes place. This separation of assays is done via solid phase adsorption, liquid phase adsorption or precipitation. While on the

other side, in homogenous immunoassay the separation of immunocomplex is not required (Ullman, 2005).

1.2 Lateral flow immunoassay

An immunoassay is a simple biochemical test that measures the concentration of an analyte in a complex system like serum, saliva or urine using an antibody-antigen reaction. Lateral flows assays are a form of immunoassay often used for medical diagnostics also referred to as immunochromatographic assay. Lateral flow assays are simple procedures to detect the presence or absence of target analytes in a complex mixture and test kits have become popular in the clinical diagnostic field for biological samples such as hormones, antigens, toxins, drugs (Kim and Park, 2004). The commercially available pregnancy test kits are classic example of lateral flow immunoassay. (Osikowicz *et al.,* 1990)

A lateral flow assay is the important part of this study which is a form of "sandwich assay". The main elements of lateral flow devices are: sample profilers pad, conjugate pad, nitrocellulose membrane containing specific capture antibody for the target analytes and an absorbance pad (Figure 1.2). The lateral flow test kit usually has an inlet for receiving a biological sample. After the complex sample (blood, serum, plasma or urine) is applied to the inlet, the sample flows from the sample pad through particles (*eg*. SERS active particles, gold particles, fluorescence particles labelled with detection antibody, *etc.*). Here they mix and the specific immunochemical reaction takes place. The liquid sample continues to transit through the nitrocellulose membrane towards the test line where capture antibodies are immobilised. Depending upon the analytes present in the sample the particles may react at the test line and generate what may be a visually distinguishable colour on the band. Excess liquid will continue to flow through the nitrocellulose towards a control line that

confirms the success of the assay by forming a colour band. The rest of the fluid continues to flow and can be collected in an absorbent pad. Thus, two bands are formed on the nitrocellulose, one of which is a test line for detection of the sample by its concentration and the other is the control line (Kim and Park, 2004). In order to detect multiple analytes, more than one test line can be used. Lateral flow tests can operate in either competitive or sandwich assay formats.

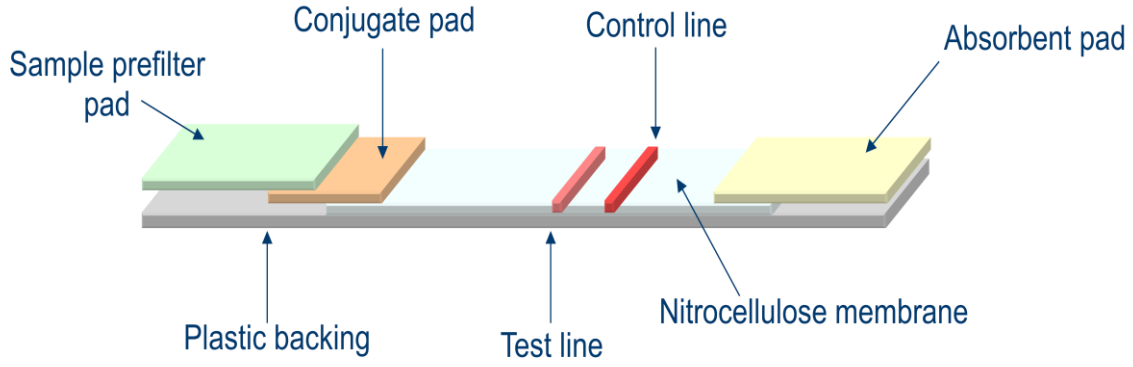


Figure 1.2: Schematic of typical lateral flow strip.

1.3 Surface enhanced Raman spectroscopy (SERS)

SERS is a surface sensitive technique. Discovered in 1970, it was shown that when small organic molecules, such as pyridine, adsorbed onto the surface of silver or gold, then the surface would exhibit 10^{6}- 10^{14} enhancement of Raman scattering (Nie and Emory, 1997; Woo *et al.,* 2009). The exact mechanism of the enhancement effect of SERS is still not clear but two primary theories are involved:

1. Electromagnetic theory arises from the presence of surface plasmons on the metal substrate. When a substrate is placed in the proximity of the Plasmon, it experiences an enhanced electromagnetic field and produces an enhanced scattered Raman field; and

2. Chemical theory that involves transfer of charge between the chemisorbed species and the metal surface. This enhancement is generally less than a factor of 10 (Stiles, 2008).

1.3.1. The Raman Effect and normal Raman scattering

When light is scattered from a molecule, most scattered photons have the same energy (frequency) as the incident photons. However, a small fraction of light (approximately 1 in 10^7 photons) is scattered at optical frequencies different from, and usually lower than, the frequency of the incident photons. The process leading to this inelastic scatter is termed the Raman Effect. Raman scattering can occur with a change in vibrational, rotational or electronic energy of a molecule. The difference in energy between the incident photon and the Raman scattered photon is identical to the energy transferred to the scattering molecule. The energy of the vibration, rotational or other mode depends on the molecular structure and environment. Atomic mass, bond order, molecular geometry and hydrogen bonding all affect the vibrational force constant which, in turn dictates the vibrational energy (Stiles, 2008).

SERS has enabled the sensitive detection of a variety of compounds of forensic, environmental or medical interest. The enhancement potentially enables detection of very low concentrations of biomolecules. The SERS technique also has the strong capability for multiplex targeting using individual or combinations of spectral signatures (Zavaleta *et al.,* 2009). SERS technology works in five steps: (1) laser light is shone on to a metal substrate; (2) plasmons are excited in the metal surface; (3) incident light is scattered by molecules absorbed on the metal surface; (4) Raman scattered light is transferred back to plasmons and scattered in air; and (5) plasmons at the metal surface act as antennae, which assist in coupling light into molecules close to the surface and couple out photons in specific

directions (Le Ru and Etchegoin, 2009). It is coupling into and out of the molecule that enhances the Raman signal (Figure 1.3).

The plasmon response depends on the molecule's structure and environment and the Raman spectra of different molecules are unique. Therefore SERS technologies leverage advantages like:

1. Sensitivity due to metal enhancement of the Raman signal;
2. Multiplex capability through the use of various dyes to produce tags with unique spectra (Bishnoi *et al.,* 2011);
3. Ability to detect and quantitate in complex mixtures, for example blood;
4. Fast detection;
5. Handheld instrumentation;
6. Compatibility with lateral flow device manufacturer and calibration.

These advantages and its highly specific nature, mean that Raman has become a very powerful tool for analysis and chemical monitoring (Baena and Lendl, 2004). Depending on the instrumentation, it can be a unique technique used for the analysis of solids, liquids and solutions and can even provide information on physical characteristics such as orientation or crystalline phase.

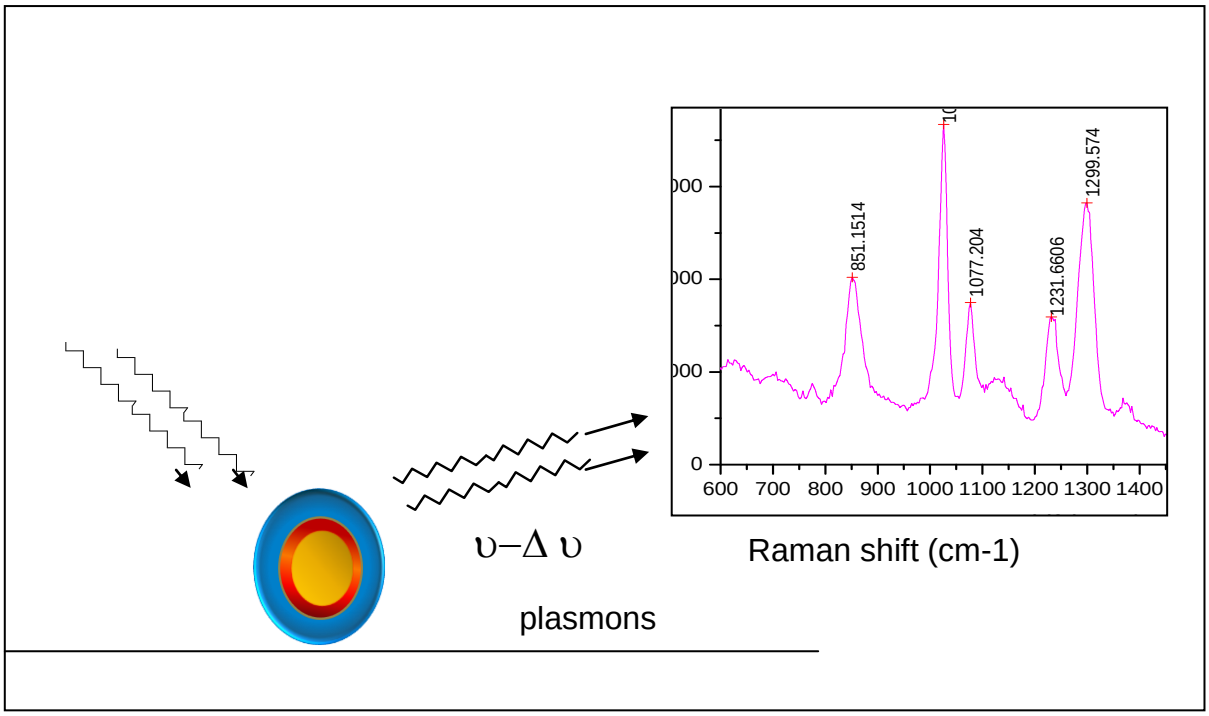


Figure 1.3: Schematic principle of surface enhanced Raman spectroscopy (SERS). When laser light scatters on the surface enhanced Raman scattering (SERS) molecule, signal enhancement arises via interaction of inelastic scattered light with surface plasmonas.

1.3.2. SERS Particles

Surface enhanced Raman scattering (SERS) active particles are developed by Oxonica Ltd and consist of a central gold core covered with a reporter molecule which is then encapsulated in silica to prevent the molecule from diffusing off the surface and, also, to avoid non-specific interactions of biological samples with the SERS active molecule (Figure 1.4).

To produce antibody conjugates the silica coating is chemically activated to enable specific attachment of an antibody to create a relevant bio-tag conjugate. Each of these Raman active particles gives a Raman spectrum dependent on the SERS reporter molecule.

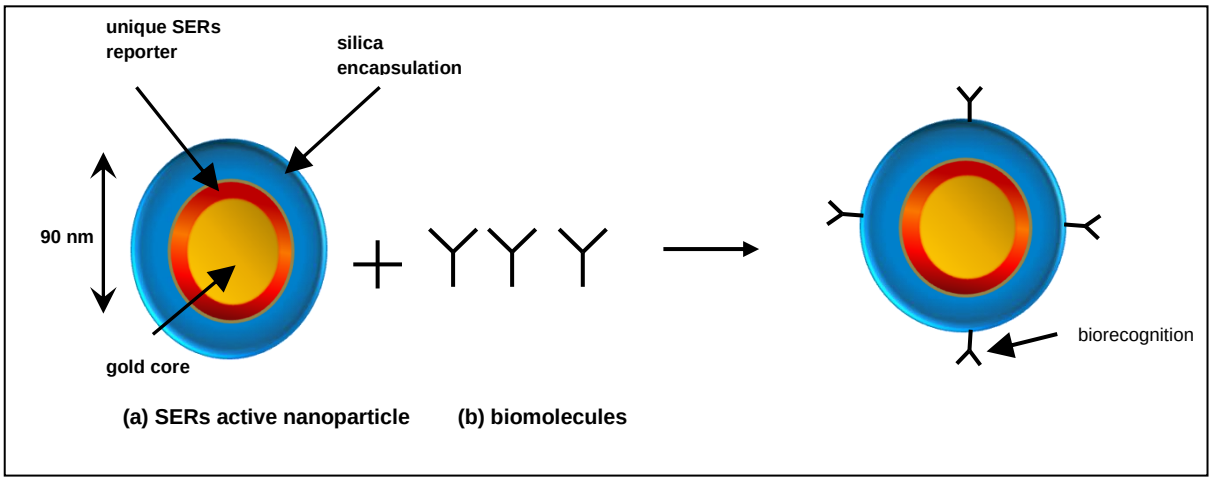


Figure 1.4: Antibody conjugations with surface enhanced Raman scattering (SERS) active nanoparticle. The gold core is covered with a unique SERS reported molecule and then with a silica shell, which can be labelled with antibodies via chemical coupling to create a particle that specially interacts with the target analyte.

1.3.3. SERS particles on lateral flow assay

This study utilizes SERS active particles on lateral flow strips and uses Raman spectroscopy to detect the particles. Specific antibodies and target analytes were obtained from commercial suppliers. Techniques like enzyme linked immunosorbent assays (ELISA), dynamic light scattering (DLS) and UV-Vis spectroscopy were used to characterise the conjugate of the antibodies and SERS particles. This allowed for the identification of the bioactivity and stability of the conjugates.

Lateral flow strip analyses are performed to detect multiple analytes by drying SERS active particles and antibodies on to the nitrocellulose strip. Parameters like blocking buffer effect capture antibody-loading concentration analysed for quality control analysis. Each analyte is detected singly over its pathological range using the lateral flow format.

SERS based lateral flow immunoassay and quantitative optical detection technology provides these potential advantages over existing lateral flow assays currently on the market.

- Multiplexing capability by using different SERS active particles with different specific biological attachment. This will be used in a single test line in a sensitive lateral flow immunoassay format. Such an arrangement is not possible with other optical tags due to cross reaction or quenching of signals between different tags;
- Leveraging of the performance of SERS active nano-particles to potentially increase sensitivity 1000 times over standard gold nano-particle bio-conjugates;
- SERS active nano-particles have a convenient, glass/silica-encapsulated format that allows simple bio-functionalization with proteins and nucleic acids with near IR detection to minimize sample background interference. Devices can therefore be read in the presence of whole blood and other biological matrices.

1.4 Selected target analytes

Multiple analytes with a market need and clinical relevance in a POC test were identified in order to demonstrate multiplexed tests. Acute myocardial infarction (AMI) represent one of the major causes of premature death in the UK (35%) and is one of the cases where POC testing could provide better management leading to advantages for patients and the NHS. For this reason cardiac markers are one of the major growth areas for POC testing. (Collinson, 2000; Zhu *et al.,* 2011)

When heart muscle is damaged, cell walls break down, permitting proteins and enzymes, normally contained within the cell to leak into the bloodstream. These substances are called cardiac markers. They can be detected in blood samples with specialized immunoassays. For early detection of AMI, serum high-sensitivity cardiac troponin (hs-cTnI) and myoglobin (Myo) have been shown to be important biomarkers. (McCord *et al.,* 2001; Plebani and Zaninotto, 1998)

This study has chosen the most common cardiac markers used in the evaluation of chest pain and acute myocardial infarctions that are troponin-I, myoglobin and C-reactive protein (CRP).

1.4.1. Troponin I

Troponin plays an important role in cardiovascular muscle contraction and regulation of skeletal muscle. Human cardiac troponin complex is 52kda. Troponin consists of three protein sub-units: troponin I (23kda), troponin T (35kda) and troponin c (18kda) which bind to the thin filament (actin) of striated muscle. Its major regulatory function is to bind calcium and regulate muscle contraction. Following injury to muscle cells (heart or skeletal muscles) the intact troponin complex along with free troponin subunits are released into blood (Thobhani *et al.,* 2010; Perry, 1979). Although troponin is found in both skeletal muscles and the myocardium, the amino acid sequences for cardiac troponin T (cTnT) and I (cTnI) isoforms are significantly different from their skeletal muscle counterparts. Commercial assays have been constructed for measurement of cTnT and cTnI in blood that have been shown to have high specificity for cardiac disease. These assays are highly sensitive because the tissue concentrations of cardiac troponin T and I are higher than for other cardiac markers. The normal concentrations of these cardiac proteins are much lower than for myoglobin, CRP and CK-MB, as these proteins are also released from normal skeletal muscle turnover (de Winter et al., 1995). Cardiac troponin I is used as an aid in the diagnosis of myocardial infarction since its concentration becomes elevated in the blood approximately four to eight hours following myocardial injury or necrosis and remains elevated for several days (Ahammad *et al.,* 2011). The pathological range of troponin I is 0.05 – 30 ng/ml and the 'cut-off' (elevated level for cardiac infarction) is 0.4 ng/ml (Zhu *et*

al., 2011). Cut off levels are those concentrations accepted as thresholds at or above which the test is called positive as below which is called negative. Further in this study troponin I is identified as troponin in simple term.

1.4.2. Myoglobin

The second most important analyte is Myoglobin. Myoglobin is a protein (18 kDa) that transports oxygen in muscle tissue, including the myocardium and skeletal muscle. Myoglobin concentration in blood rises within one to two hours from the AMI occurrence (Mauro *et al.,* 2005). It is a sensitive marker for muscle injury making it a potential marker for heart attack in patients with chest pain. Myoglobin as a cardiac marker, discharge quickly from the damage cell than other cardiac marker. This is an advantageous for earlier detection of AMI (Lewandrowski *et al.,* 2002). The pathological range is 5 - 500 ng/ml with a cut off limit of 107 ng/ml (Penttila *et al.,* 2002).

1.4.3. C-Reactive protein

C - reactive protein is found in the blood stream and the concentration rises in response to inflammation. In acute inflammation it rises above normal limits within six hours and peaks at eight hours. C - reactive protein is mainly associated with liver failure. In a recent study it was found that subjects with elevated levels of C-reactive protein were at increased risk of diabetes, hypertension and cardiovascular disease (Dehghan *et al*., 2007; Clearfield, 2005). A recent study shows that CRP is co-deposited with other activated complement within all acute myocardial infarcts (Lagrand *et al.,* 1997; Algarra *et al.,* 2013). Additionally, CRP peaks at the highest values at around 48 hours after the onset after myocardial infarction (Pepys and Hirsecfield, 2003; de Beer *et al.,* 1982). Therefore this study has chosen C-reactive

protein as a third analyte for multiplexing assay. Pathological range of C-reactive protein is 0.1 – 3 μg/ml with a cut off limit of 3 μg/ml (HyTest Ltd, 2013).

1.5 Technology used for quality control analysis

Different techniques like dynamic light scattering and Ultraviolet–visible spectroscopy used for SERS particles or conjugate characterisation analysis.

1.5.1 DLS (Dynamic light scattering)

Dynamic light scattering (DLS; sometimes referred to as photon correlation spectroscopy or quasi-elastic light scattering) is a technique for measuring the size of particles typically in the sub-micron region. DLS measures the scattering of light from particles in solution. As the intensity of the light scattered is proportional to the six power of the particle size, larger particles will give a greater signal than smaller particles. Thus a small percentage of aggregates will have a large intensity signal compared to a large proportion of small particles. A major advantage of the DLS technique is that samples can be quickly analysed in solution to determine whether the conjugates or the SERS itself single or in mixture are aggregated or not.

1.5.2 UV-Vis (Ultraviolet–visible spectroscopy)

Ultraviolet–visible spectroscopy (UV-Vis) refers to absorption spectroscopy or reflectance spectroscopy in the ultraviolet-visible spectral region. It uses light in the visible and adjacent (near-UV and near-infrared (NIR) ranges. The absorption or reflectance in the visible range directly affects the perceived colour of the chemicals involved. Molecules containing π-electrons or non-bonding electrons (n-electrons) can absorb the energy in the form of ultraviolet or visible light to excite these electrons to higher anti-bonding molecular

orbitals. The more easily excited the electrons (i.e. lower energy gap between the HOMO and the LUMO) the higher the wavelength of light it can absorb.

CHAPTER 2: Materials and Methods

2.0 Introduction

This chapter will have a list of all the materials used in the whole study. It will then explain the method of antibody loading on the SERS particles and different techniques used for their characterisations. After that this chapter will conduct the establishment of the sample preparation for the single and mixture lateral flow strips using the capture antibody. Finally the method of preparing, cutting and running a lateral flow assay will be demonstrated at the end.

SERS instrumentation and Strips running techniques are also covered in this section.

2.1 Materials

Thiolated glass coated gold colloid particles (Au/glass) SERS 420, SERS 421, SERS 440 and SERS 470 were purchased from Becton Dickinson, (Chicago IL, USA). Rabbit anti-mouse antibody for the control line, human CRP free serum, human troponin, myoglobin and C-reactive protein and their respective monoclonal antibodies (MAb) were obtained from HyTest Ltd (Turku, Finland). The heterobifunctional cross linker, succinimidyl 4-[N-maleimidomethyl] cyclohexane-1-carboxylate (sulfo-SMCC), Tween 20 and 0.5 ml gel filtration columns were purchased from Thermo Scientific (United Kingdom).

Absolute ethanol and Parafilm M were purchased from Fisher Scientific (Loughborough, Leicestershire, UK). Anti-mouse IgG (whole molecule) antibody raised in rabbits was purchased from Sigma Aldrich (Poole, Dorset, UK). Other materials to prepare buffer and assist the conjugation process and lateral flow strip preparation were purchased from Sigma-Aldrich (Poole, Dorset, UK). Polyester backed nitrocellulose UniSart CN-140 (25 ×

50m) was purchased from Whatman Ltd. (Maidstone, Kent, UK). Plastic backing cards (60 mm × 300 mm) for nitrocellulose mounting were purchased from BioDot Ltd. (Chichester, East Sussex, UK).

Listed below are the buffers most commonly employed throughout the study. Water supplied from a Milli-Q Millipore 'Advantage' water purifier was used throughout.

Conjugation Buffer: 50 mM borate at pH 6.5 for myoglobin and at pH 7.2 for troponin and CRP conjugations (pH adjusted by adding NaOH to boric acid solution.)

Storage/suspension buffers prepared by adding 0.1 % BSA, 0.05 % Tween 20 and 0.05 % sodium azide to the respective conjugation buffer and pH adjusted to 6.5 or 7.2 accordingly.

Coating buffer: 20 mM potassium phosphate, 2 % D-sorbitol, and 2 % ethanol, pH 7.4

Running buffer:100 mM borate, 2 % BSA and 5 % Tween 20 pH 7.5.

Buffers were supplemented with additional detergent (Tween 20) as required. All solutions were filtered through a 0.2 % filter prior to use.

2.2 Conjugation of protein to SERS nanoparticles

Monoclonal detection antibody (M19C7) anti-troponin (stock concentration 3.2 µg/ml) was diluted in borate buffer pH 7.2 to make up final concentration of 15 µg/ml. The diluted antibody solution was added into a fresh solution of sulfo-SMCC in water (2 mg/ml, 6.5 µl) and allowed to react 45 minutes. The final volume was 2.5 µl. borate buffer (40 µl) was added so as to make up 65 µl volume to use in filtration column. Unreacted cross linker was removed using micro bio-spin chromatography columns following the manufacturer's directions using borate buffer (Thermo Scientific Pierce, 2013).

The column's bottom closure was removed and the column placed in 1.5-2.0 ml microcentrifuge collection tube and centrifuged at 1500 g for one minute to remove the

storage solution. The column was placed in new collection tube and 300 µl of buffer added and the column equilibrated. This step was repeated three times and the column placed in a new centrifuge tube. 30-130 µl of antibody solution was slowly applied to the centre of the compact resin bed and filter and centrifuged. Purified antibody was collected in centrifuge tube and desalting column was discarded after use.

Purified and maleimide activated antibody solution was added into thiol activated SERS active nanoparticles (150 µl of stock; n.b. OD of stock was ≈ 0.25 at 540 nm at 100 fold dilution) solution. SERS particles –antibodies conjugates were prepared according to the scheme shown in Figure 2.1. This solution was left on a roller mixer (Stuart SRT6) three to four hours to react and finally the reaction between the modified antibody and the nanoparticles was quenched by addition of 2-mercaptoethanesulfonic acid sodium salt (1 mg/100 µl, MESA). After quenching 45 minutes the conjugates were purified by centrifuging (5 min, 1000 RCF) on a micro centrifuge (Eppendorf). Supernatant was discarded and the pellet resuspended in resuspension buffer (215 µl; 20 mM borate buffer, 0.1% BSA, 0.05% Tween 20, 0.05% sodium azide). The centrifugation/resuspension step was repeated four times and finally the particles were resuspended in buffer (50 µl) and stored in a refrigerator prior to use.

Monoclonal detection antibody (C6) anti-CRP conjugation with SERS nanoparticles was carried out using the same method and buffer as troponin conjugation. But for CRP antibody loading on SERS particles was 500 µg/ml. Monoclonal detection antibody (7C3) anti-myoglobin conjugation with SERS nanoparticles was also carried out with the above conjugation procedure in borate buffer at pH 6.5 and the final antibody loading on particle was 270 µg/ml. Myoglobin conjugates were stored in suspension buffer at pH 6.5.

For optimisation of the conjugation method, each of the conjugate was prepared in four different buffers - sodium borate, sodium phosphate sodium acetate and PBS using three different pH 6.5, 7.2 and 7.5. Using the above method each conjugate was prepared and visually optimised for aggregation. Results are shown in the chapter 3. Optimised conjugates were used for the further study.

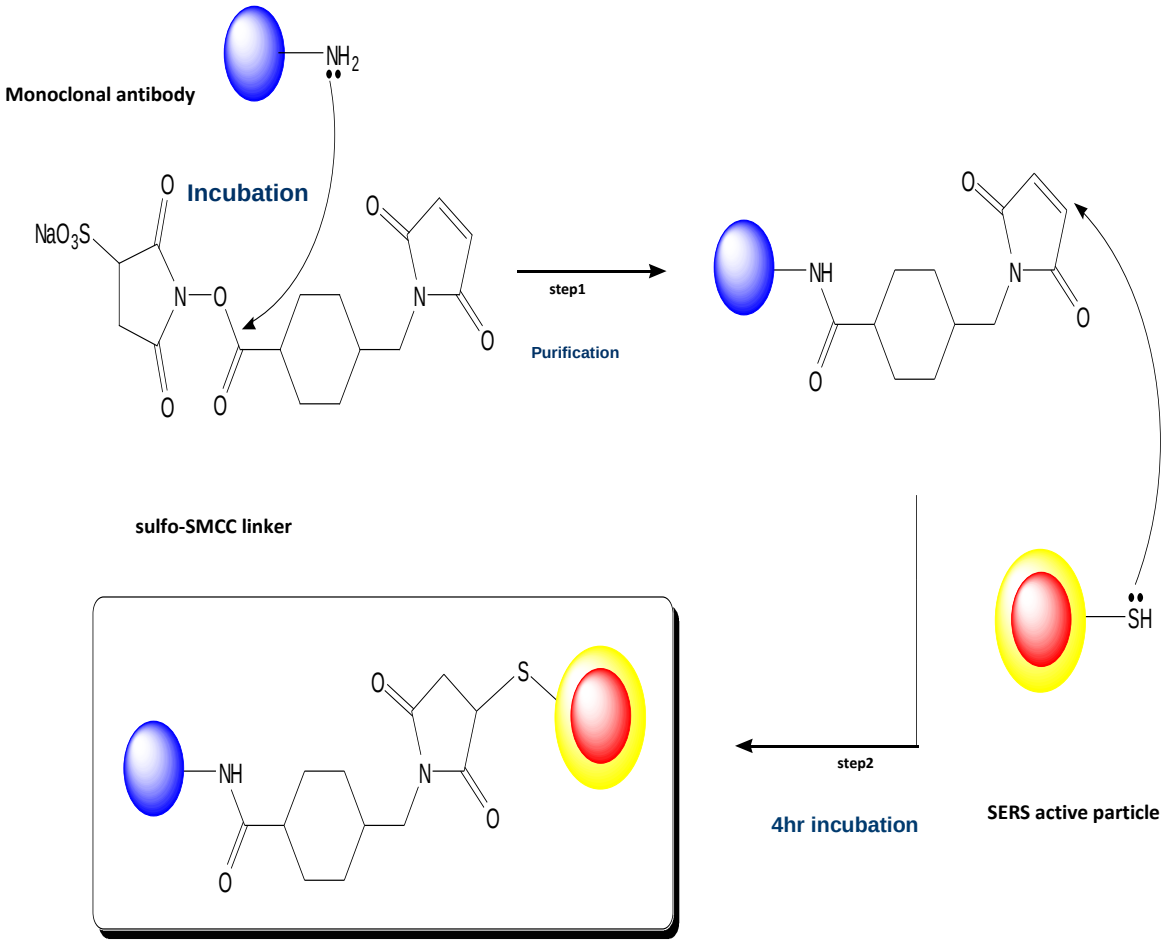


Figure 2.1: Reaction sequence for preparation of antibody–SERS particle conjugates. In the first step of conjugation, the NHS ester of sulfo-SMCC is reacted with lysine residues on the carrier protein, converting them to reactive maleimides that can react with thiolated SERS active particles and form antibody–SERS particle conjugates.

2.3 Fabrication of lateral flow strip using of monoclonal antibodies

MF4 and 560 capture monoclonal antibodies for troponin were purchase in stock concentration of 6 mg/ml and 12.6 mg/ml. From stock 1 mg/ml of MF4 and 560 were prepared in coating buffer and deposited on nitrocellulose strip CN140 using the Biodot

system. Thickness of test line is roughly 1.1 mm. Similarly, 1 mg/ml of 4E2 myoglobin capture antibody and 0.6 mg/ml of C2 antibody were used to prepare single analyte lateral flow strips for myoglobin and CRP assays. Troponin capture antibody 1 mg/ml, myoglobin capture antibody 1 mg/ml and 0.6 mg/ml CRP capture antibody were mixed in a tube with coating buffer prior speeding on the nitrocellulose for multiplexing assay.

For the control line in a single and multiplex strip, rabbit anti-mouse antibody (1 mg/ml) was prepared from the stock of 3.4 mg/ml and loaded on each nitrocellulose strip at a distance of 4 – 5 mm from the test line. Each nitrocellulose strip was kept in the oven at 37 °C for half an hour. Each strip was mounted on a plastic backing card before cutting in 5 mm width using a biodot cutter (Biodot Ltd, U.K) and stored in separate boxes at 4 °C.

2.4 Biodot system

A batch of lateral flow strip is prepared by Batch Dispense Workstation (XYZ 3050) and Cutting System (CM4000) supplied by Biodot. A batch Dispense Workstation (XYZ 3050) contains Dispense Ranges: Frontline, Biojet and Airjet. Frontline has minimum line width of 0.33 mm, BioJet spray 20 nL - 2 µL/cm while AirJet spray 1 µL/cm - 22 µL/cm. It has a dispensed area of 450 × 70 mm. All dispensing is controlled by a handheld controller. The cutting system (CM4000) has safety interlock device as well.

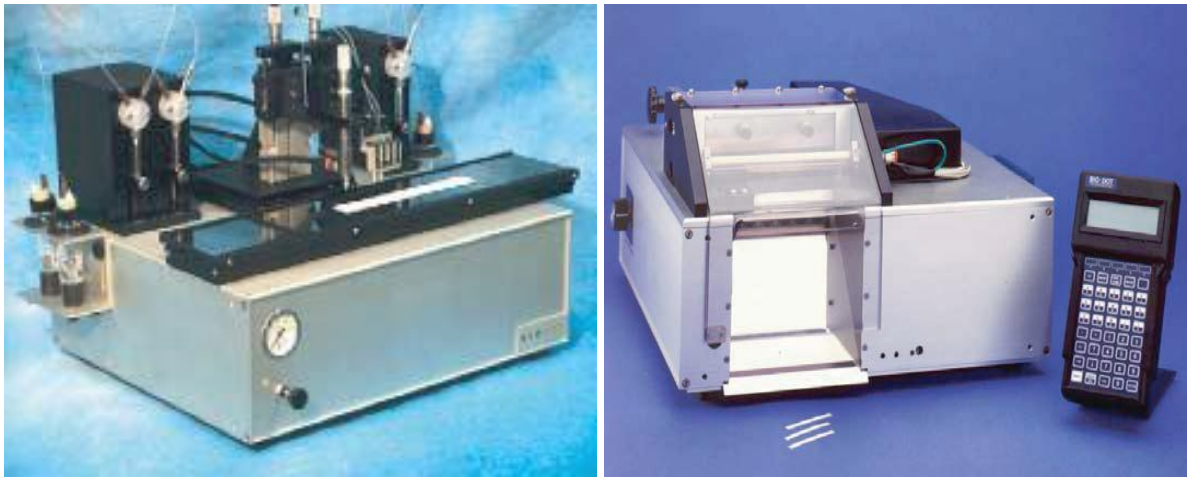


Figure 2.2: Batch Dispense Workstation (XYZ 3050) for preparing lateral flow strips and Cutting System (CM4000) for cutting strips

2.5 Sample preparation and lateral flow assay running method

100 ng/ml troponin was prepared from troponin stock (heart complex) 1000 ng/ml in 50 µl of CRP free serum. Similarly 1000 ng/ml myoglobin was prepared from stock of 1mg/ml and 10 µg/ml of CRP was prepared from 100 µg/ml of stock. Stock aliquots were frozen at -80°c. For the single assay each analyte was prepared in a serial dilution in eppendorf tubes using CRP free serum. 1 µl of each conjugate was added in respective analyte dilution and mixed well before running on the lateral flow strip.

While for the multiplex assay the top concentration of each analyte (50 ng/ml for troponin, 500 ng/ml myoglobin and 5 µg/ml CRP) was prepared in 50 µl CRP free serum. Serial dilutions were performed. The initial multiplexing assay was performed using an equal mixture of conjugate (5 µl of each conjugates) were mixed and 1 µl of this mixture was used in the assay (Results are in chapter 4 sections 4.1). Further in multiplexing myoglobin and troponin conjugate addition was altered by increasing their volume. 5µl CRP conjugate and 7.5 µl of myoglobin and troponin conjugate were mixed in separate tube. Total 20 µl of conjugates having ratio of 1:1.5:1.5. In each dilution 1ul of conjugate mixture was added.

2.6 SERS particles

Four different SERS active particles were identified for the study. Each particle is differentiated by the nature of the SERS reporter molecule immobilised on the gold core. The particles were selected on the basis of the spectral features unique to the reporter - thus enabling the particles to be differentiated from each other (Figure 2.3).

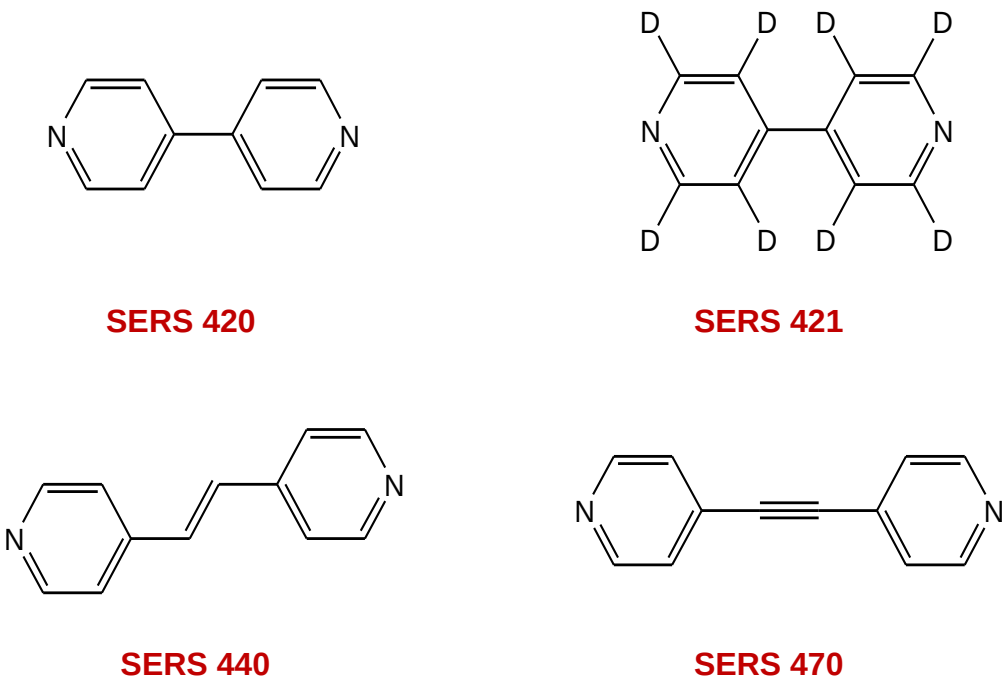


Figure 2.3: SERS active molecules identified for use in this study. Product name is shown beneath each molecule.

2.7 SERS instrumentation

The Raman System R-3000 QE (Ocean Optics, Dunedin, USA; Figure 2.4) instrument is a partially integrated analyser for real-time quantitative and qualitative analysis of aqueous solutions, powders, tablets, gels and surface media. It includes a CCD-array spectrometer with a wavelength range of ~200-2700 cm^{-1} and 785 nm (red) solid-state diode lasers. The probe head coupling the sample to the spectrometer and laser is a flexible fibre optic cable (100 µm diameter and with one-meter length). Fibres are protected with stainless steel

sheathing. The stainless steel sampling tube is 3.5 inches long and 1/2 inch in diameter. There are two focusing caps: one focuses the laser beam on solid samples as the cap is pressed against the sample on lateral flow strip. The other focuses the laser on the inside of transparent containers containing liquids or gels when the cap is pressed against the outside wall of the container. To measure liquid sample liquid cap was used. Additionally, for instrument calibration acetonitrile was used this allowed for quick, software-controlled instrument calibration and verification.

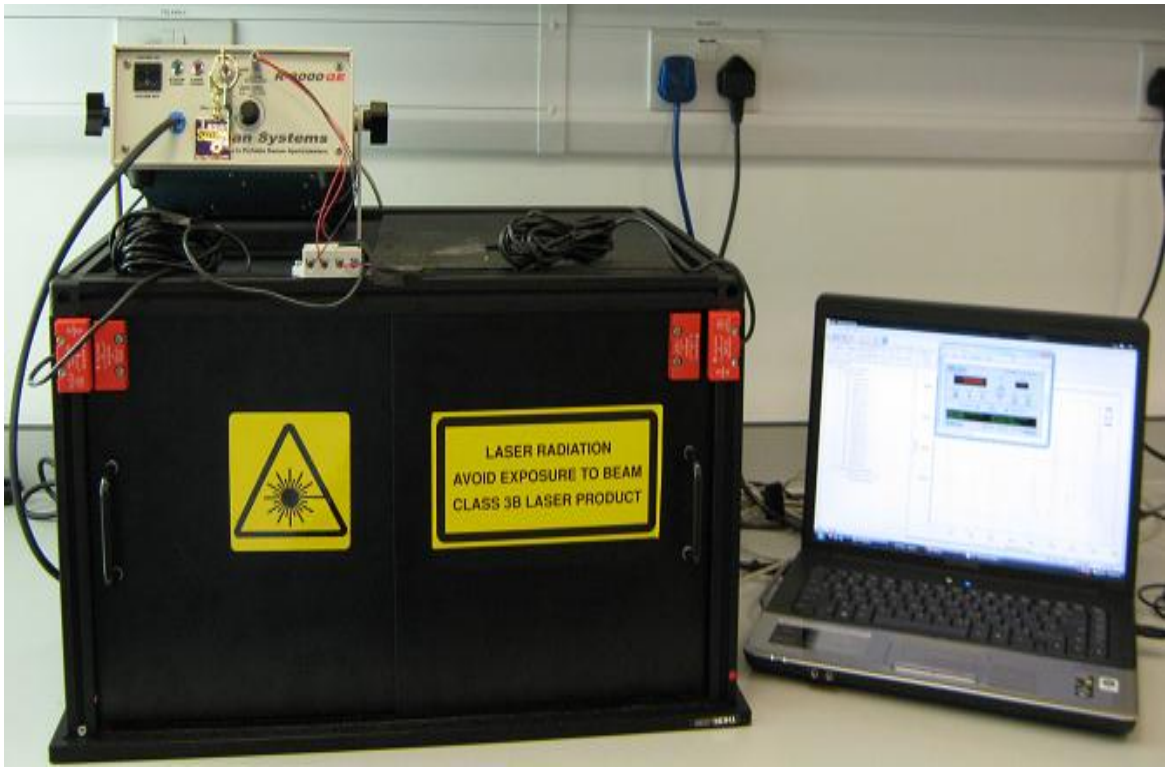


Figure 2.4: Raman spectrometer system with interlock.

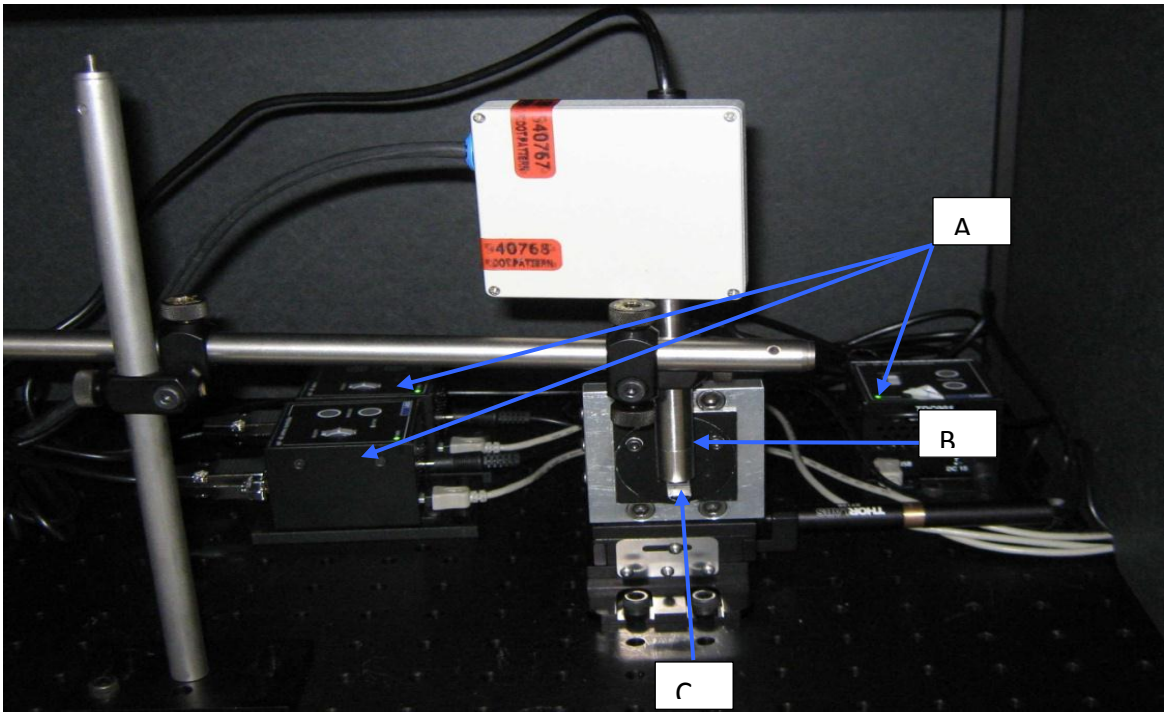


Figure 2.5 Ocean Optics reader assembles with black safety box and interlock system. Internal view of black box that contains three-stage controller, laser probe and strip holder.
A: Stage controllers (x, y and z control)
B: Fibre optic probe 100-micron focal point 4.6 mm focal length
C: Strip holder on x, y and z stage (± 40 nm resolution)

The laser beam emerging from the R-3000 laser output port or from the fiber optic probe is a Class 3b laser. This laser product produces visible and/or uv laser radiation capable of causing serious eye injury and blindness to anyone who looks directly into the beam or its specular reflection. Therefore some modifications were done for the safety purpose. A black box with an interlock system was built (Figure 2.4). The box door was connected with magnetic interlock. So, when the door is open the shutter is shut down. In order to hold the lateral flow strip and measure via probe a strip holder was designed, built and fixed to an *x, y* and *z* stage controller controlled via software computer (Figure 2.5). Also the laser probe was fixed to the stage inside the black box. Lateral flow strips were cut in a fixed size and held by the strip holder at the same position every time. The laser scanning mode could be

adjusted by control software (RSI, Renishow). Two different scanning procedures were evaluated using SERS nanoparticles strips. SERS particles are diluted 2, 4,8,16 and 64 fold in eppendorf tubes and then spread on the lateral flow strip using a biodot system. All strips were scanned by the Raman reader using two methods. The first scanning method scanning was 3 different positions on a test line and each point was exposed 30s by laser and collected data. The 2nd way of strip scanning was 3times across Y-axis at 37 mm/s. linear cam can scan 1.1 mm test line with constant velocity. This can cover only a small background and whole test line across y axis. Data was collected in csv format and peak height and peak area were analysed by Ranishow software .Results are shown in chapter 3 (section 3.3).

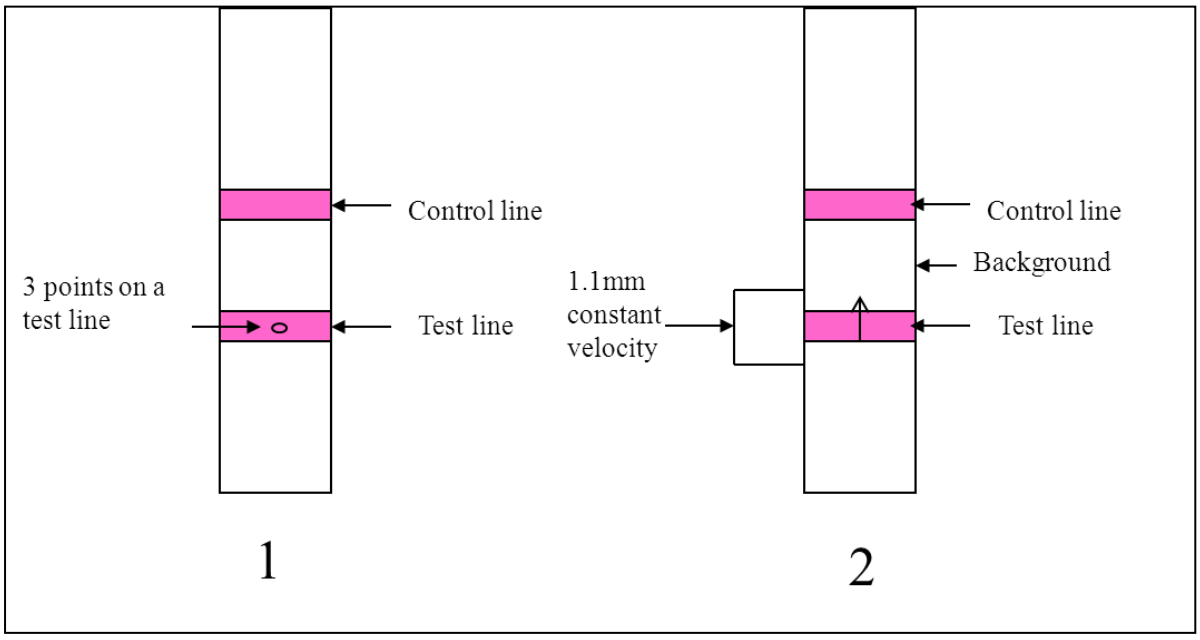


Figure 2.6: 1st strip is scanning three points on a test line. And 2nd strip is scanning Y axis (from bottom to top of the test line) at 37 mm/s using linear cam.

2.8 DLS (Dynamic light scattering)

Measurements were performed on the SERS particles to confirm the size of each particle and visualize the aggregation in buffer. Also SERS conjugates measurements were

performed to optimise different pH and varying protein-loading concentrations. All light scattering measurements were carried out using a Zetasizer Nano ZS (Malvern DTS 5.1). The viscosity of the sample was assumed to be the viscosity of the dispersant. Measurements were carried out at 25 °C in disposable cuvettes using a sample volume of 100 μl. Each sample was diluted 10 fold and measured in triplicate and the mean value reported. The wavelength of the HeNe laser used in the DLS instrument was 633 nm.

2.9 UV-Vis (Ultraviolet – visible spectroscopy)

UV-Vis measurements were carried out using a Lambda 850 spectrophotometer (Perkin Elmer) using a standard 1 cm path length quartz cuvette from Optiglass Ltd (Essex, UK). The reference beam sample was water or appropriate buffer. Spectra were obtained from 200 to 800 nm (spectral resolution 1 nm), with the lamp change occurring at 319.2 nm. Milli-Q water was used as the blank for SERS particles and conjugate buffer used for the conjugation reactions. SERS nanoparticle solutions were monitored for both conjugation and aggregation by measuring SERS solution with: no antibody, immediately after addition of the antibody, at the end of the reaction and after centrifugation and re-suspension of the conjugates in storage buffer. Absorbance spectra were measured for each nanoparticle and their respective antibody-conjugates between the wavelengths of 800 nm to 200 nm. UV-Vis measurements can be used to indicate adsorption of proteins onto SERS with a change in absorbance peak for the nanoparticle.

2.10 Two run assay

In order to find steric hindrance in the assay two run assays was designed where first sample will run and then second sample will apply on the strip so if the first sample is

blocking the sights on the test line it will not allow binding of the second analyte from the second run. Multiplexing strips with troponin, myoglobin and CRP capture antibody strips were used for the assay. Troponin was used as model. Therefore run 1 was either CRP OR Myoglobin. Therefore for run 1, CRP 11µl (9900 ng/ml) mixed with 22µl of serum and 3.3µl of sample buffer were mixed with 1.1µl of CRP conjugates or without conjugates were adjusted by 4.4 µl of buffer and run on the strip. Similarly myoglobin (1620 ng/ml) was mixed with 22µl serum and 3.3 µl buffers as mention above. For run 2 troponin (150 ng/ml) was mixed with 22µl of serum, 1.65 µl of troponin conjugate and 2.75 µl of sample buffer. Sample buffer was adjusted to keep the loading volume the same in each assay.

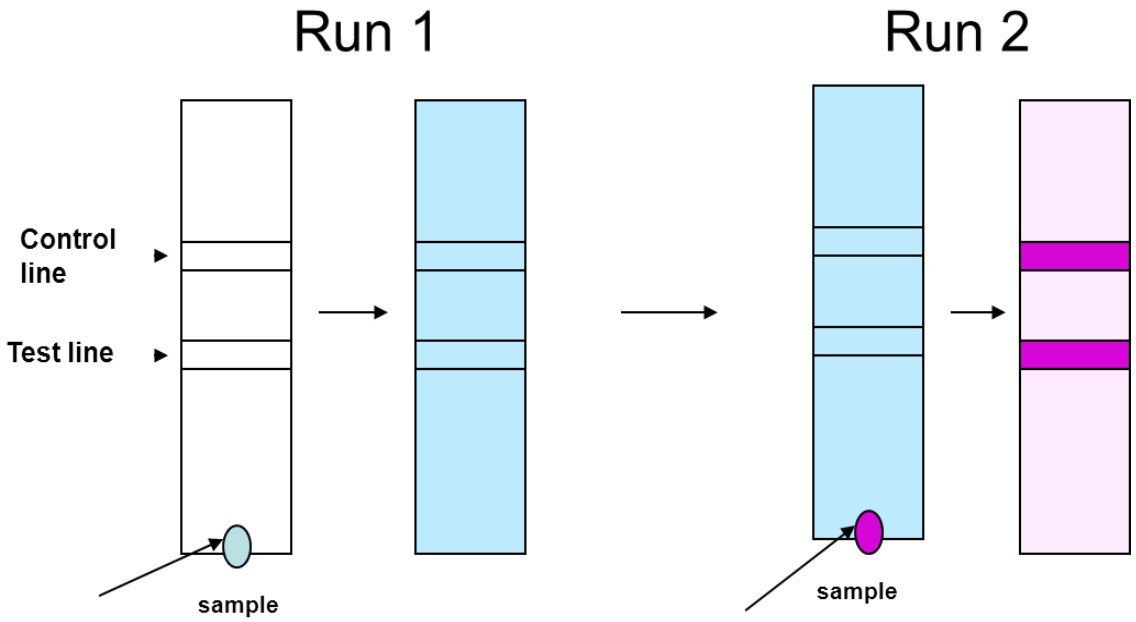


Figure 2.7: In run 1 first analyte or buffer is running single form then in run 2 another analyte will be run on the same strip. Here CRP and myoglobin effect on troponin is analysed and troponin is run most of the time as a run 2.

2.11 Effect of high, medium and low concentration of analytes in multiplexing

To investigate high, medium and low effect of each analytes on others candidates, three concentrations were selected for each. Table 2.1 and 2.2 represent the selected concentration of respective analytes. Multiplexing strips were prepared as mentioned in section 2.5. Total twenty seven strips were run in duplicates for this assay. Sample volume and sample running method was kept the same as previously mention in section 2.5.

Analyte	High(ng/ml)	Medium(ng/ml)	Low(ng/ml)
CRP	1800	200	21
cTnI	50	5	0.54
Myo	178	30	6.5

Table 2.1: High, medium and low concentration of all three candidates.

Strip No	CRP	Myoglobin	Troponin
1	High	High	High
2	High	High	Medium
3	High	High	Low
4	High	Medium	High
5	High	Medium	Medium
6	High	Medium	Low
7	High	low	High
8	High	low	Medium
9	High	low	Low
10	Medium	High	High
11	Medium	High	Medium
12	Medium	High	Low
13	Medium	Medium	High
14	Medium	Medium	Medium
15	Medium	Medium	Low
16	Medium	low	High
17	Medium	low	Medium
18	Medium	low	Low
19	low	High	High
20	low	High	Medium
21	low	High	Low
22	low	Medium	High
23	low	Medium	Medium
24	low	Medium	Low
25	low	low	High
26	low	low	Medium
27	low	low	Low

Table 2.2: CRP, troponin and myoglobin concentration in each strip.

CHAPTER 3: Calibration and characterisation

3.0 Introduction

The purpose of this study was to explore the use of surface enhanced Raman scattering (SERS) particles in an immunoassay for developing an innovative concept of multiple analyte detection on a single test strip. This chapter was aimed at: (1) characterisation and selection of the SERS particles for multiplex measurements; (2) development of the laser scanning procedure for reading the Raman signal from the SERS particles on the test line of lateral flow strips; (3) preparation and evaluation of SERS particle - antibody conjugates and (4) development of simplex lateral flow assays using each of the three selected analytes - troponin, myoglobin and CRP. Each of these is described in detail below.

(1) Characterisation and selection of the SERS particles for multiplex measurements:

Numerous studies have shown that the multiple characterisation techniques are required for the development of good-quality immunoassay conjugates to achieve enhanced performance of the diagnostic test (O'Farrell, 2009; Thobhani *et al.*, 2010). Therefore, various characterisation techniques such as Raman analysis, UV-visible (UV/Vis) absorption spectroscopy, dynamic light scattering (DLS), were used to determine the extent of SERS particles' nature, aggregation, size, shape, stability in different media and activity of the biological molecule (antibody) on their surfaces.

Four SERS particles labelled with 420, 421, 440 and 470 reporters (structure of these reporter molecules is shown previously in Figure 2.3) were purchased from Oxonica. Raman spectrum of each SERS particles (420, 421, 440 and 470) was collected by a Raman reader. All Raman spectra were overlaid to identify a unique Raman peak for each SERS particle. This unique Raman peak was then used as the signature for identifying a particular SERS

particle. Additionally, two different combinations of three SERS particles were prepared to identify the combination giving the best well-separated unique Raman peaks (section 3.1). A calibration analysis was carried out to determine the relationship between the Raman intensity and concentration of each SERS particle in a suspension (section 3.2). UV-Vis analysis and DLS were used to measure the surface absorbance spectra, concentration, aggregation and size in single and mixture form of the SERS particles (section 3.4 and 3.5).

(2) Development of the laser scanning procedure for reading signal from the SERS particles on test line of lateral flow strips:

The Raman system R-3000 QE infra-red laser (Ocean Optics, Dunedin, USA) is commonly used in surface studies. This Raman system was chosen for this study because its laser frequency (785 nm) has little background interference from the blood-based samples, whilst providing a reasonable Raman signal. This laser was mounted onto a specially configured lateral flow stage holder and was operated with an interlock for safety reasons. The system was controlled using a Ranishow software to read the Raman spectra of SERS particles mounted on the lateral flow strip. A precise, stable, fast and reproducible technique of scanning the lateral flow strip to achieve maximum Raman intensity signal from a wider range of concentration of single and mixed particles bound on test line was developed after the modification of the purchased instrument. Two different scanning methods were tested by the Raman reader. The first method was to scan at three different positions on a test line, with 30 s laser exposure at every position. The second method was to scan the Y-axis three times, from bottom to top of the test line (1.1 mm scan), at a constant velocity (37 mm/s). SERS 420 particles were used as a model for this test. The Raman peak height and area were measured to choose the best scanning method for single and multiplex lateral flow research (section 3.3).

(3) Preparation and evaluation of SERS particle - antibody conjugates:

Background research have shown that the development of a good quality antibody labelling technique is essential to achieve maximum bioactivity in the immunoassay (Aubin-Tam and Hamad, 2008; Chun, 2009). Therefore, the aim of this examination was to prepare high quality and stable protein-SERS particles conjugates without any aggregation. To accomplish this several parameters such as: the molarity of the salt in reaction buffer, the pH of the reaction, the concentration of antibody loaded onto the particles, and antibody–crosslinker (sulfo - SMCC) ratio, were optimised.

The protocol of the manufacturer of crosslinker (sulfo - SMCC) suggested that the buffer selection should be made by avoiding buffers containing primary amines (e.g., Tris or glycine) and sulfhydryl during conjugation, because they can compete with the intended reaction. A pH below 7.5 for the maleimide group stability and the ratio of protein to crosslinker (5 - 80 - fold molar excess) were also recommended by the manufacturer's protocol (Thermo Scientific Pierce, 2013). Therefore, to obtain ideal conditions, individual SERS – detection antibody conjugates were prepared in different buffer and pH conditions and were optimised for aggregation first visually in solution, and then on the lateral flow strip. Similarly, antibody loading on SERS particles and antibody–cross-linker (sulfo - SMCC) ratio were also optimised (section 3.6).

(4) Development of simplex lateral flow assays using each of the three selected analytes - troponin, myoglobin and CRP:

Each biomarker was individually analysed on a single lateral flow strip using the conditions optimised in section 3.6, prior to moving on the complex system (mixture assay) to understand its performance and behaviour. Dose response curve were conducted for, troponin labelled to the 470 SERS particles, myoglobin labelled to the 440 SERS particles and

CRP labelled to the 421 SERS particles using analyte concentration proximate to their dynamic range in buffer and serum (section 3.7 and 3.8). This experiment was conducted to identify the individual measurable range of each biomarker with the SERS detection technique.

3.1 Spectra for various SERS

Raman spectra of suspensions of each SERS particle are shown in Figure 3.1 and the spectra are overlaid in Figure 3.2 so that the unique peaks selection for each particle could be clearly identified.

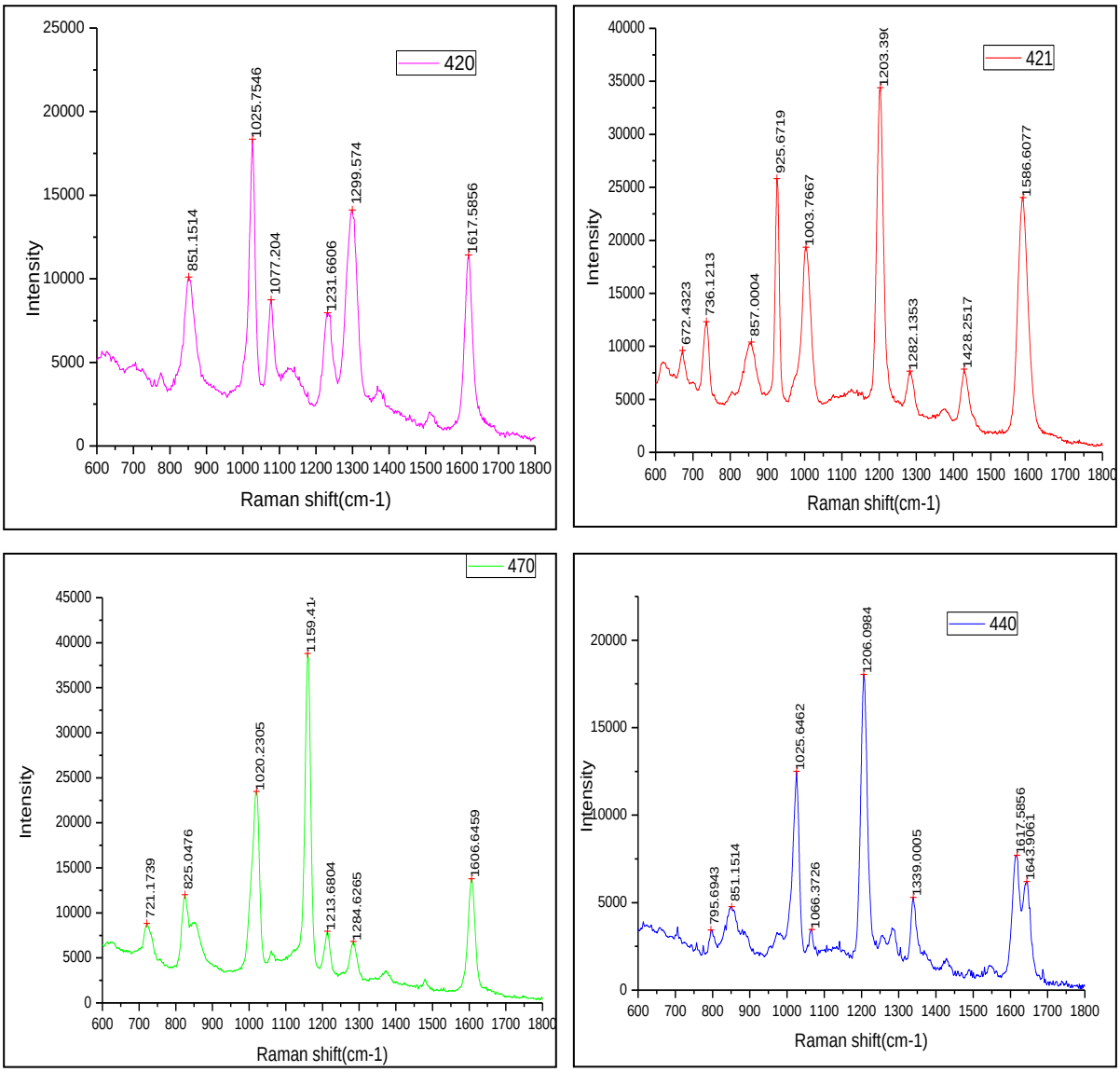


Figure 3.1: Raman spectra of the four selected SERS nanoparticles. SERS particle: (top left) 420; (top right) 421; (bottom left) 470; and (bottom right) 440.

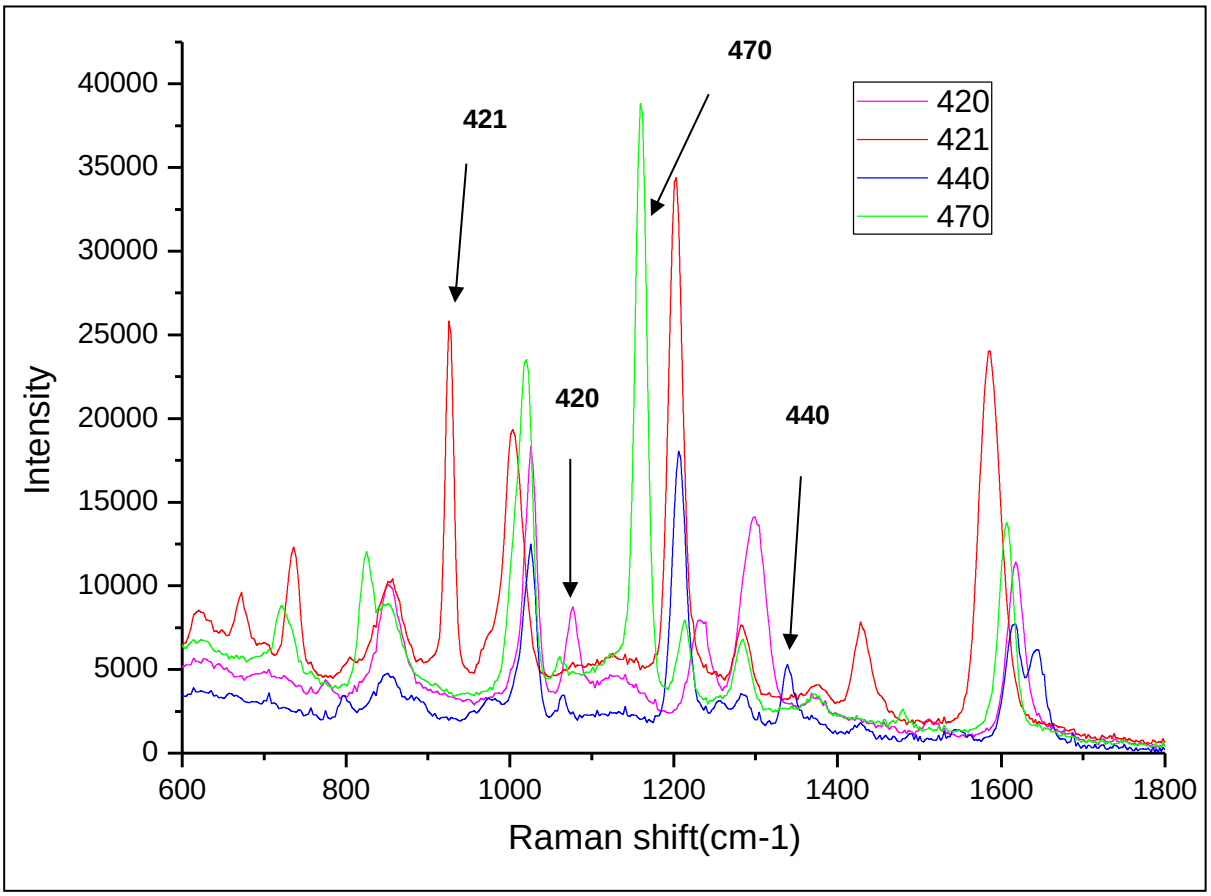


Figure 3.2: Overlaid spectra showing the peak selected to uniquely identify each particle. Here unique peak for 421 particles is at 921 cm^{-1}, 440 particle's unique peak is at 1335 cm^{-1}, 470 particle's unique peak is at 1159 cm^{-1}, and 420 particle is 1077cm^{-1}.

Overlaying of each particle gives clear indication of choosing best mixtures. Combination of 420, 421, 440 or 421, 440, 470 can be good candidates for multiplexing assay. Therefore two best mixtures (420, 421, 440 and 421, 440, 470) were prepared separately in eppendorf and spread as a line on a lateral flow strip for analysis. Results are shown in Figure 3.3 for the selected particles mixture.

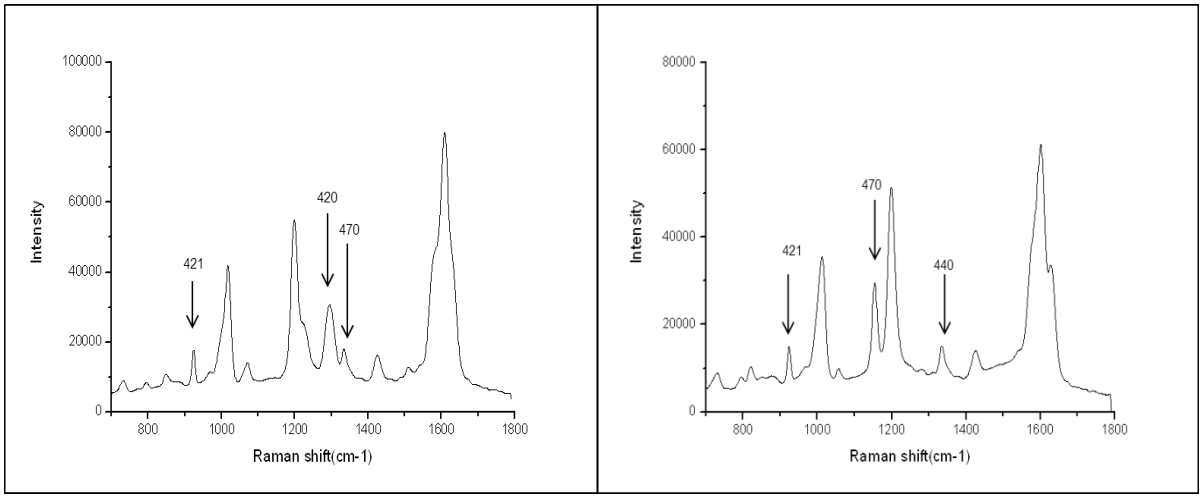


Figure 3.3: Spectrum of 2 best mixtures shows particles unique peak. (Left) 421, 420 and 470 unique peaks and (right) 421, 470 and 440 unique peak.

Two best mixtures of particles analysis showed that deconvolution of the 2nd mixture (421, 440 and 470) spectrum is clear and each particles unique peak is well separated. While in a first mixture 420 and 440 particles peaks were very close to each other and may interfere with each other for complex assay. Therefore, for the multiplexing assay the selected combination of 421, 440 and 470 particles will be used and tested for the relevant analyte.

3.2 Calibration of SERS particles in suspension

Aqueous suspension of the SERS particles were serially diluted and measured to obtain preliminary calibration plots, Figures.3.4 to 3.7.

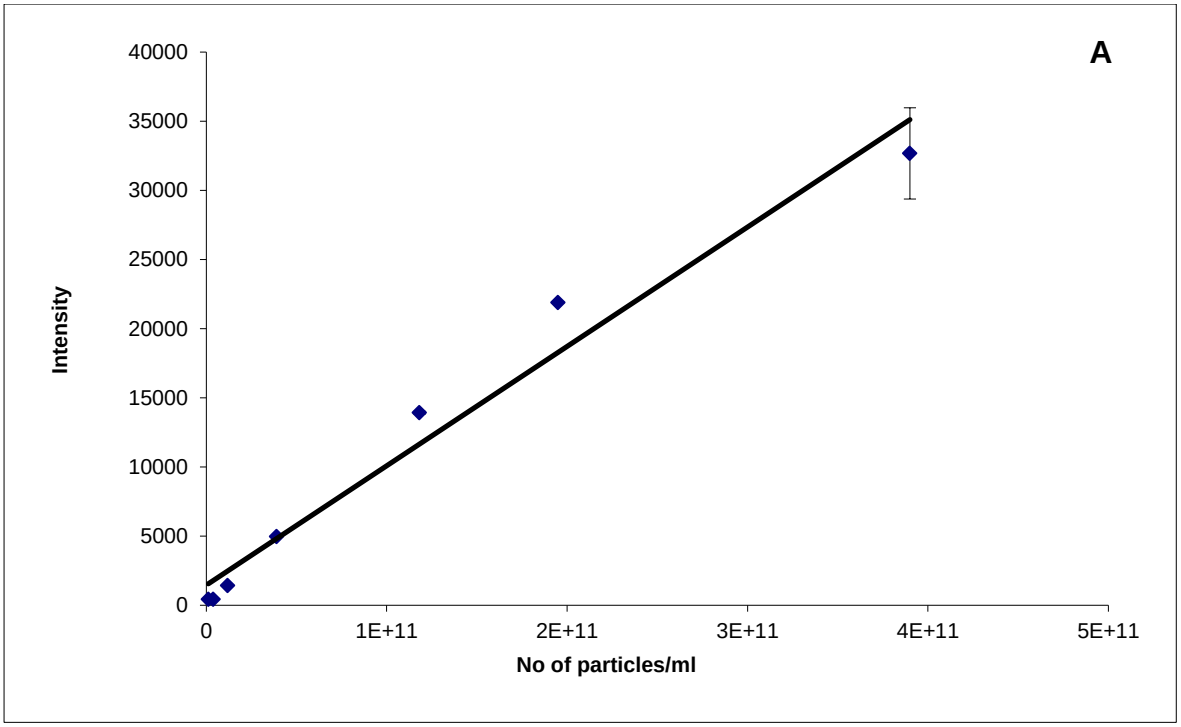


Figure 3.4: Calibration plot for Oxonica SERS 420 particles in aqueous suspension. Raman peak at 1077 cm^{-1} measured as a function of particle concentration.

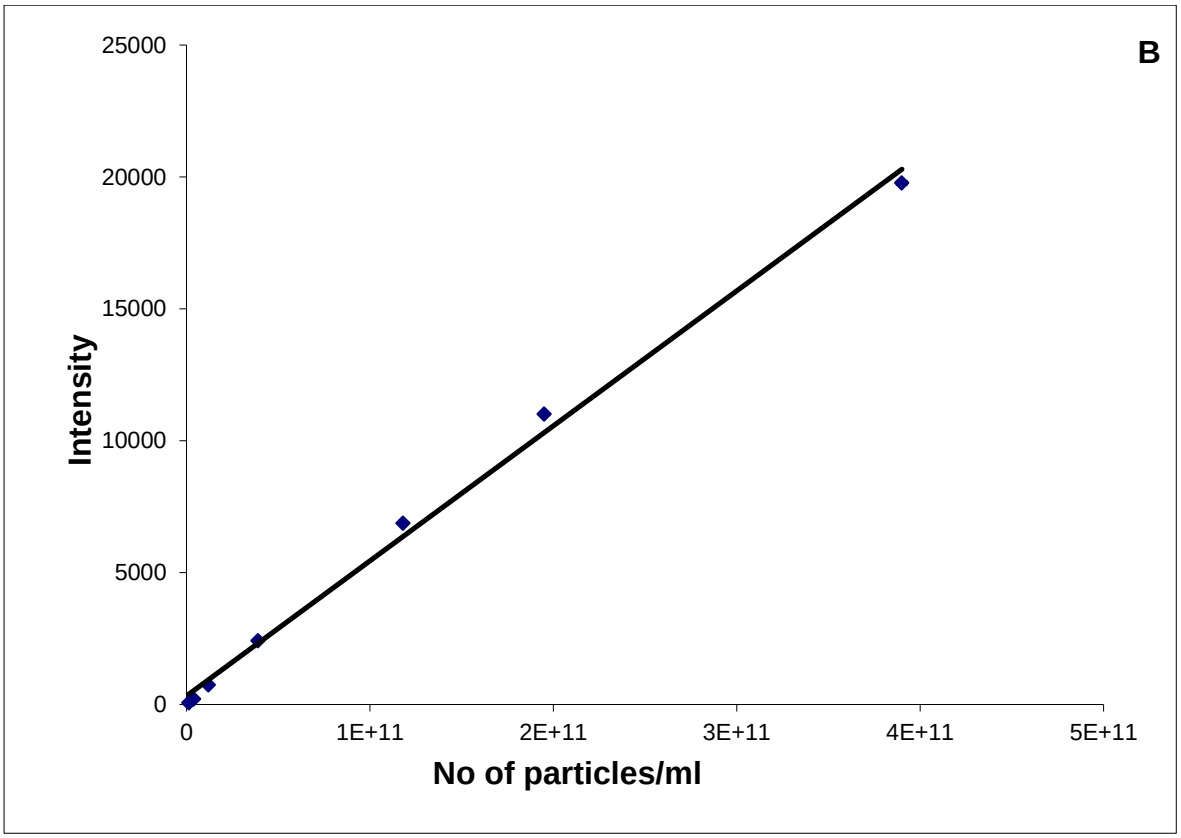


Figure 3.5: Calibration plot for Oxonica SERS 421 particles in aqueous suspension. Raman peak at 921 cm^{-1} measured as a function of particle concentration.

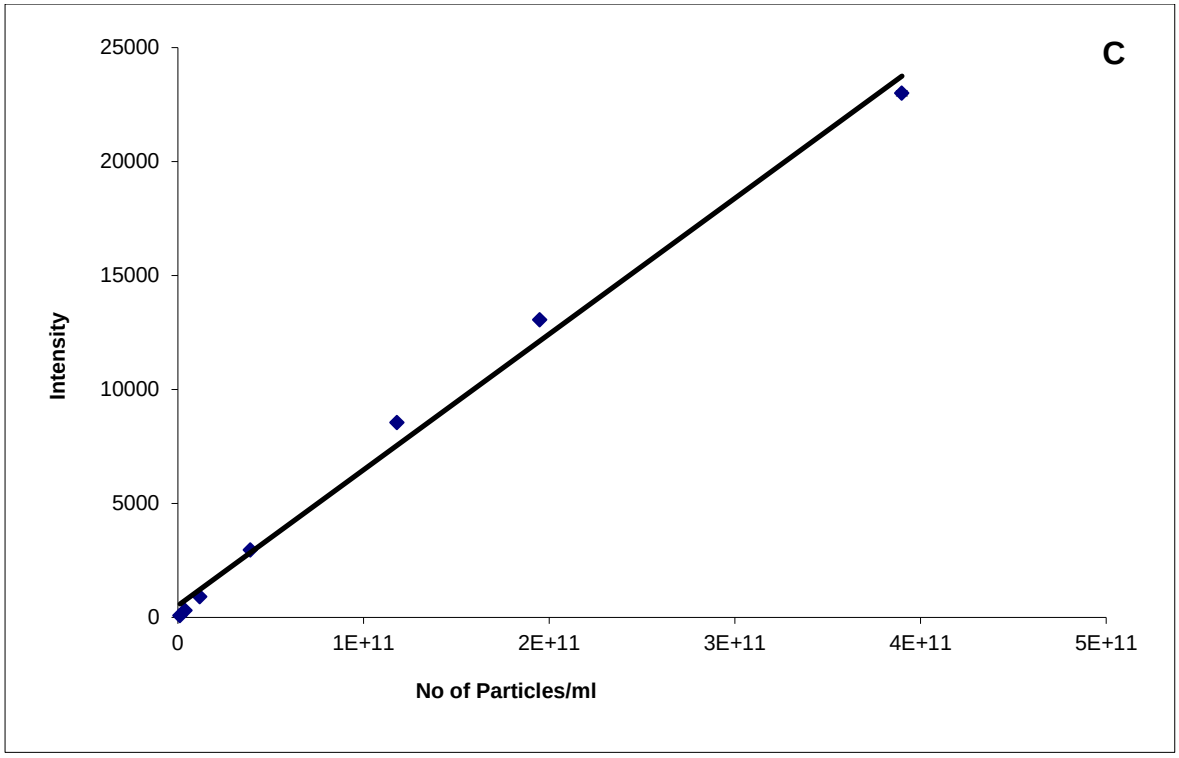


Figure 3.6: Calibration plot for Oxonica SERS 440 particles in aqueous suspension. Raman peak at 1335 cm^{-1} measured as a function of particle concentration.

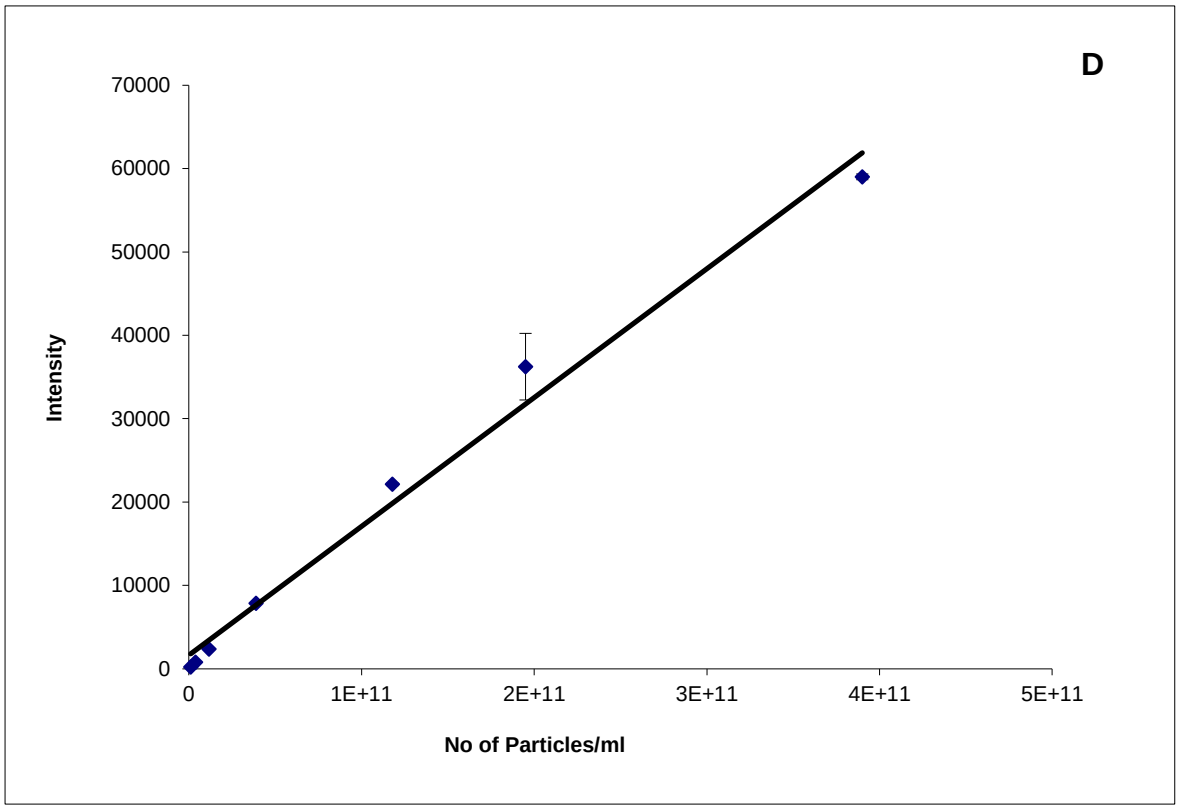


Figure 3.7: Calibration plot for Oxonica SERS 470 particles in aqueous suspension. Raman peak at 1159 cm^{-1} measured as a function of particle concentration.

The results show that there is a roughly linear relationship between the signal intensity and the concentration of particles in suspension:

$420 = (8.78E\text{-}08 \pm 6.39E\text{-}09) * [\text{SERS } 420 /ml^{-1}] + (1396 \pm 1095)$ (A)

$421 = (5.12E\text{-}08 \pm 1.43E\text{-}09) * [\text{SERS } 421 /ml^{-1}] + (324 \pm 246)$ (B)

$440 = (5.96E\text{-}08 \pm 2.21E\text{-}09) * [\text{SERS } 440 /ml^{-1}] + (521 \pm 378)$ (C)

$470 = (1.55E\text{-}07 \pm 7.99E\text{-}09) * [\text{SERS } 470 /ml^{-1}] + (1608 \pm 1369)$ (D)

3.3 Various scanning technique for lateral flow strip on Raman reader

Results of two different scanning techniques were conducted in duplicates and plotted below as Figure 3.8 using SERS 420 particles. Figure 3.8 show that 3 point scanning method doesn't give a definite response for below 0.2 % SERS particles. The average intensity of each SERS concentration is half then Y-axis scan. In summary, peak intensity and peak area of Y axis scan is clear, higher and more promising than 3 point measurement on the test line for each SERS particles concentration.

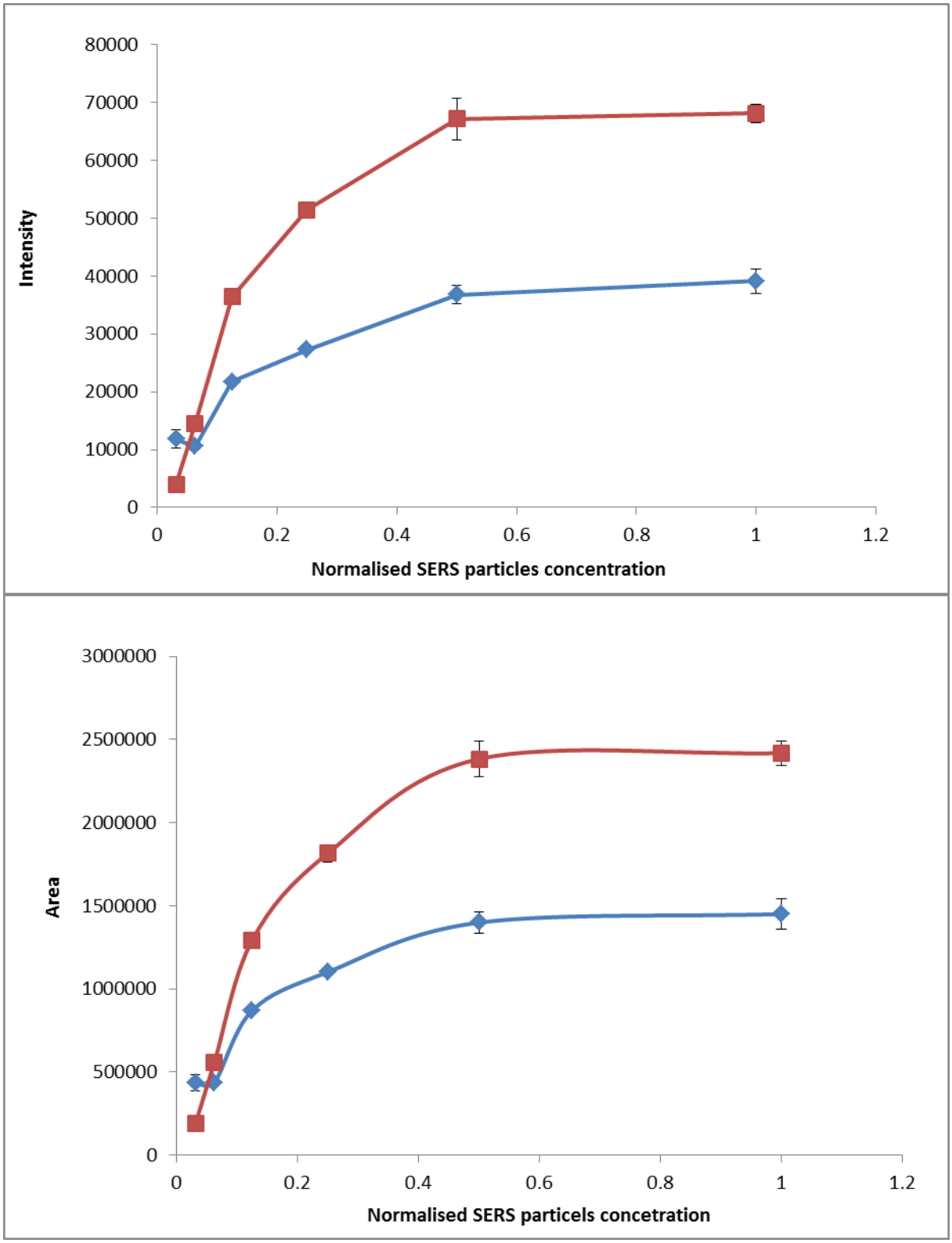


Figure 3.8: Evaluation of scanning method for measurement of SERS 420 particles on a lateral flow strip. ◆ Three point scan ■ Y axis scan

3.4 UV-Vis analysis results

All single SERS particles sequential dilution was performed in water. And each sample was measured on UV-Vis. Particle absorbance was plotted against their concentration and results are shown Figure 3.9 and Table 3.1. UV-Vis results show that all SERS particles give linear relationship between absorbance and their subsequent dilution.

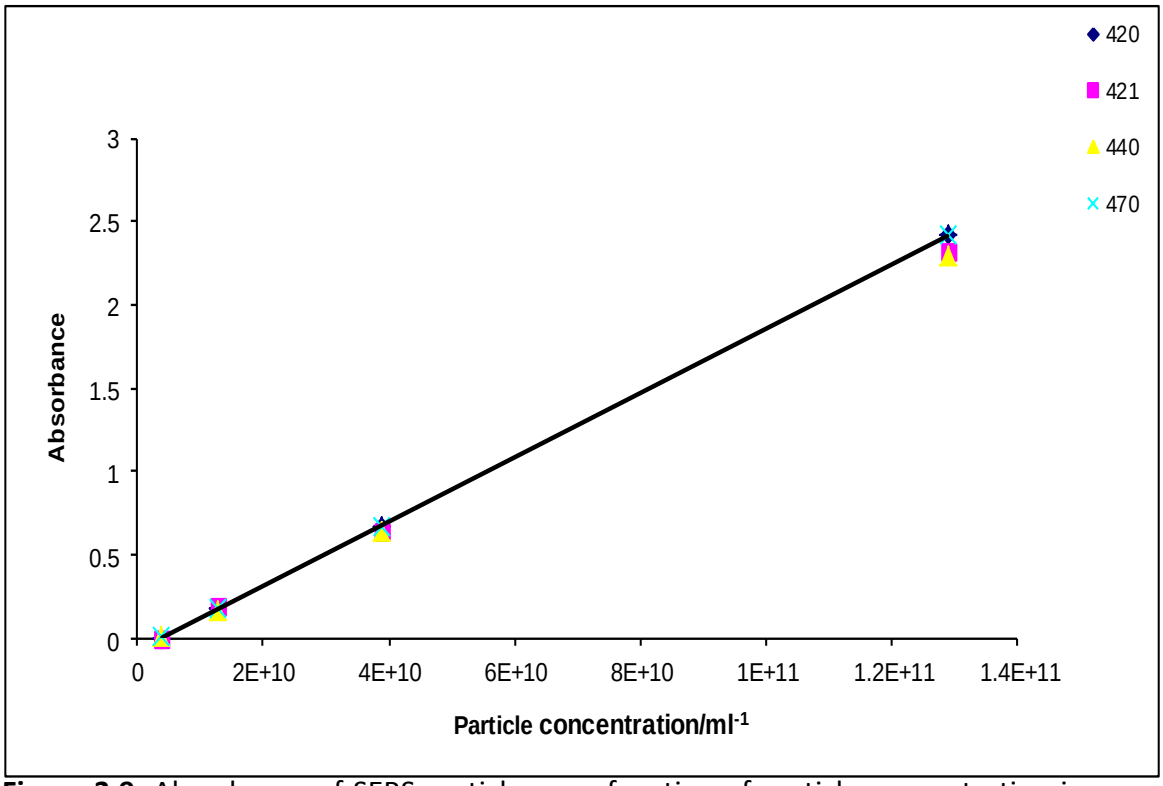


Figure 3.9: Absorbance of SERS particles as a function of particle concentration in aqueous solution. SERS particle: (♦) 420; (■) 421; (▲) 440; and (x) 470. Absorbance measured at 542 nm.

SERS Dilution	particles concentration/ml^{-1}	420	421	440	470
10	1.29E+11	2.415544	2.328781	2.289499	2.41437
33.3	39000000000	0.67276	0.646474	0.649262	0.674413
100	12900000000	0.184764	0.197418	0.171646	0.176693
333.3	3900000000	0.004379	0.005018	0.012859	0.007801

Table 3.1: Absorbance of SERS particles as a function of particle concentration in aqueous solution. Absorbance measured at 542 nm.

3.5 DLS (Dynamic light scattering) results

Size distribution measurements were performed on the single SERS particles and SERS antibody conjugates to optimise protein-loading on the surface using dynamic light scattering (Figure 3.10 to 3.13). Here intensity and volume graphs consist of a number of logarithm spaced size classes on the x-axis with the percentage of the light scattered through the relevant particles in intensity on y-axis or the volume they occupy on y-axis. Each particle shows one peak which explains the mono disparity of the particles. The presences of additional peaks were not detected indicating the absence of aggregate in the solution. Similarly, (Figure 3.14 to 3.17) the results of different combination of three selected particles (421, 440 and 470) for the further study show single peaks at ≈165-175 without showing any aggregation. Also results of these 3 selected particles conjugate (troponin - 470, myoglobin - 440 and CRP - 421) mixture was obtained by DLS and plotted in Figure 3.18. This confirms the presence of antibody in a mixture by increased size by ≈35-40nm. All measured size was in triplicates and their mean has been recorded in Table 3.2.

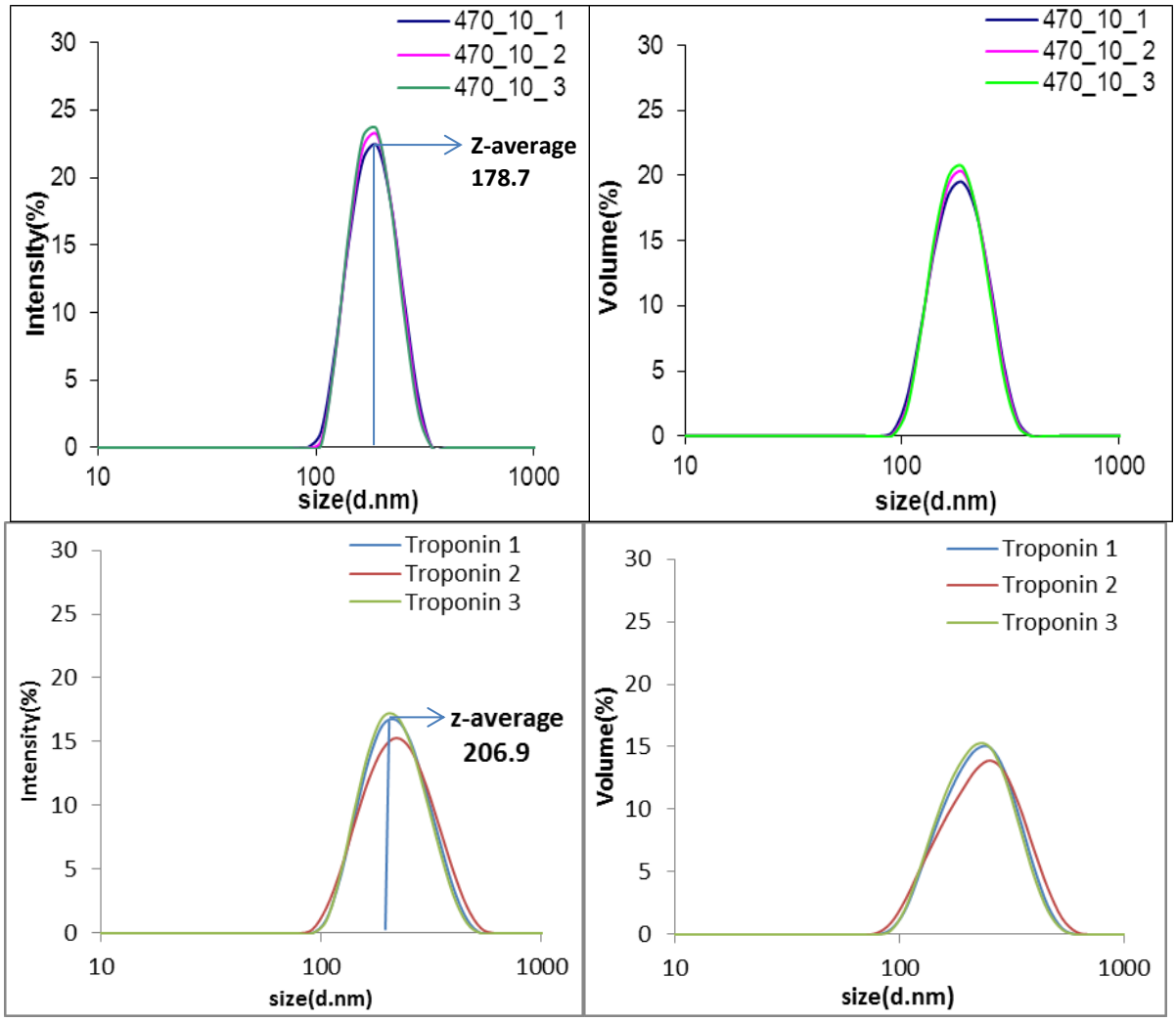


Figure 3.10: Dynamic light scattering measurements of SERS 470 particles. (Top) Naked particles - without antibody. (Bottom) SERS particles conjugated to troponin antibody. Measurements recorded in triplicate. Particles suspended in H_2O.

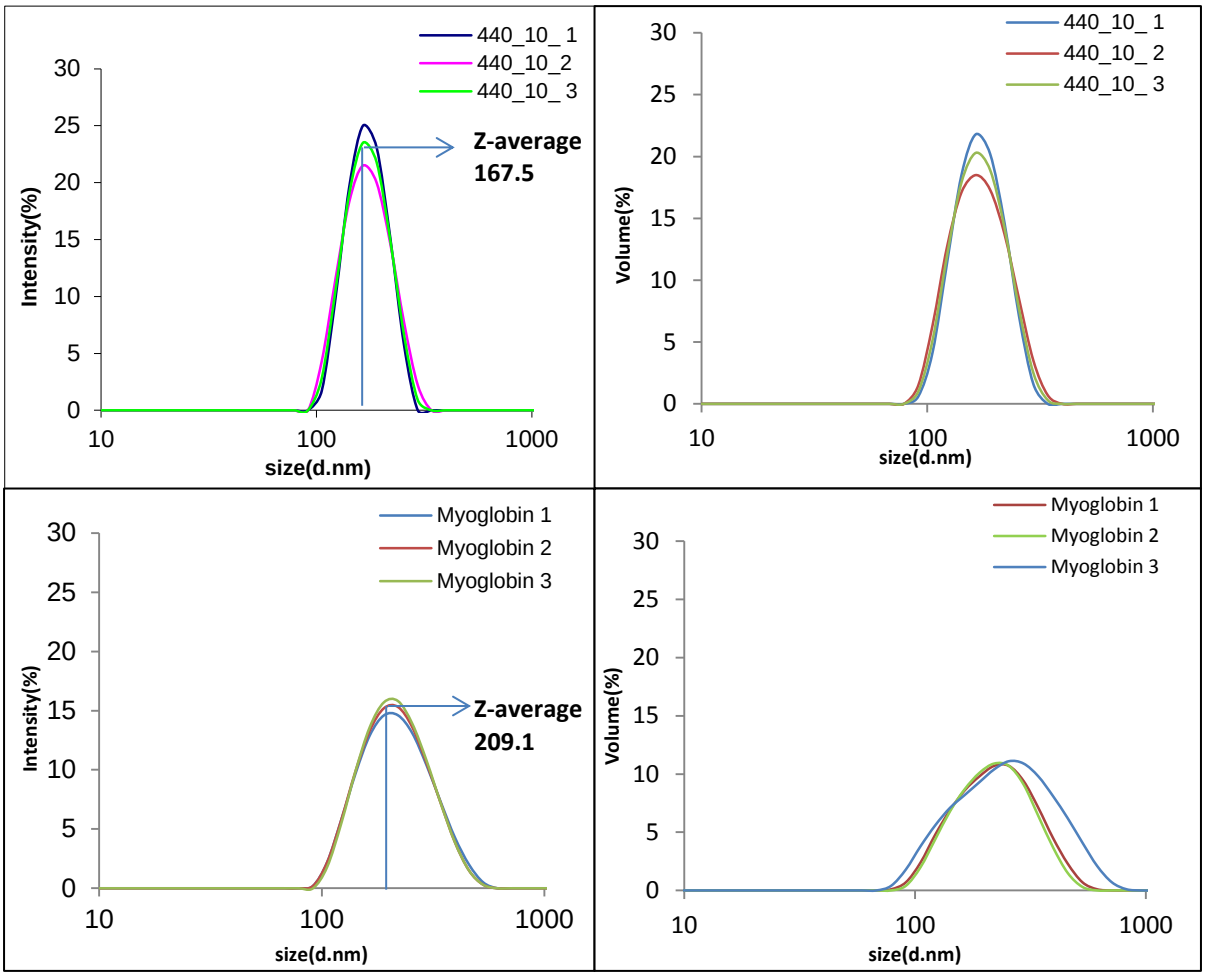


Figure 3.11: Dynamic light scattering measurements of SERS 440 particles. (Top) Naked particles - without antibody. (Bottom) SERS particles conjugated to myoglobin antibody. Measurements recorded in triplicate. Particles suspended in H_2O.

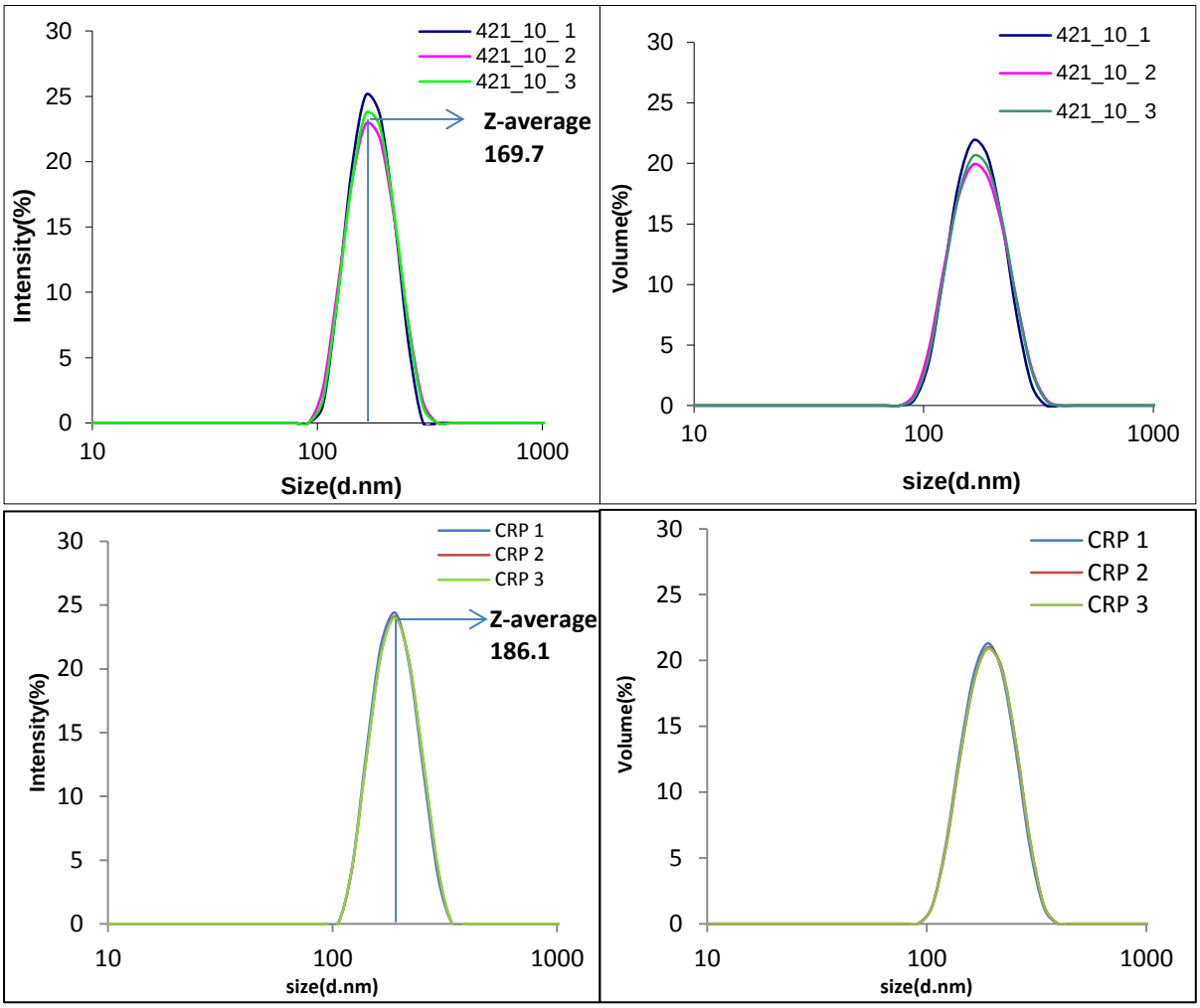


Figure 3.12: Dynamic light scattering measurements of SERS 421 particles. (Top) Naked particles - without antibody. (Bottom) SERS particles conjugated to CRP antibody. Measurements recorded in triplicate. Particles suspended in H_20.

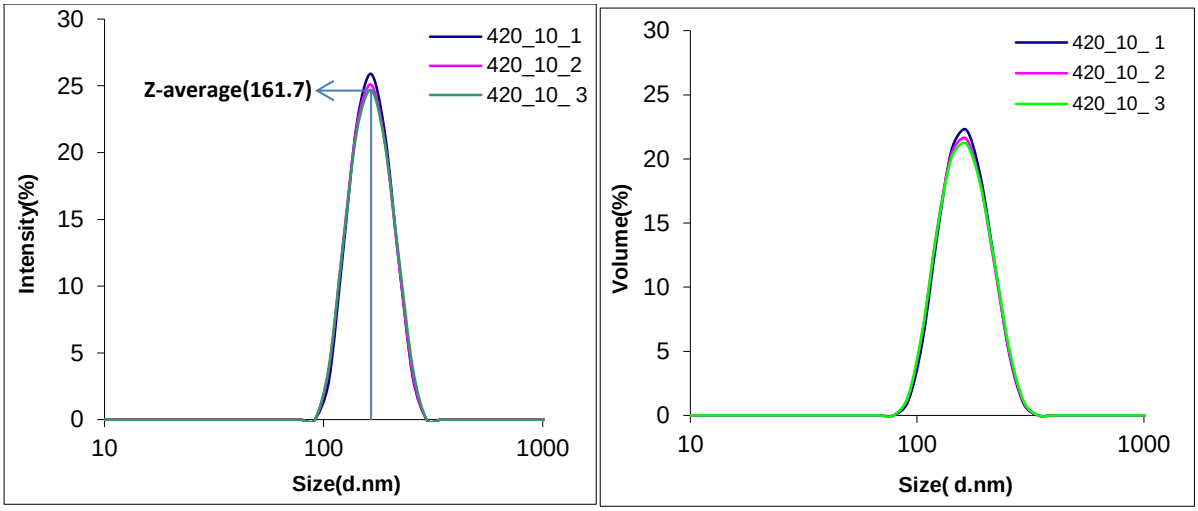


Figure 3.13: Dynamic light scattering measurements of SERS 420 particles. Only naked particles - without antibody.

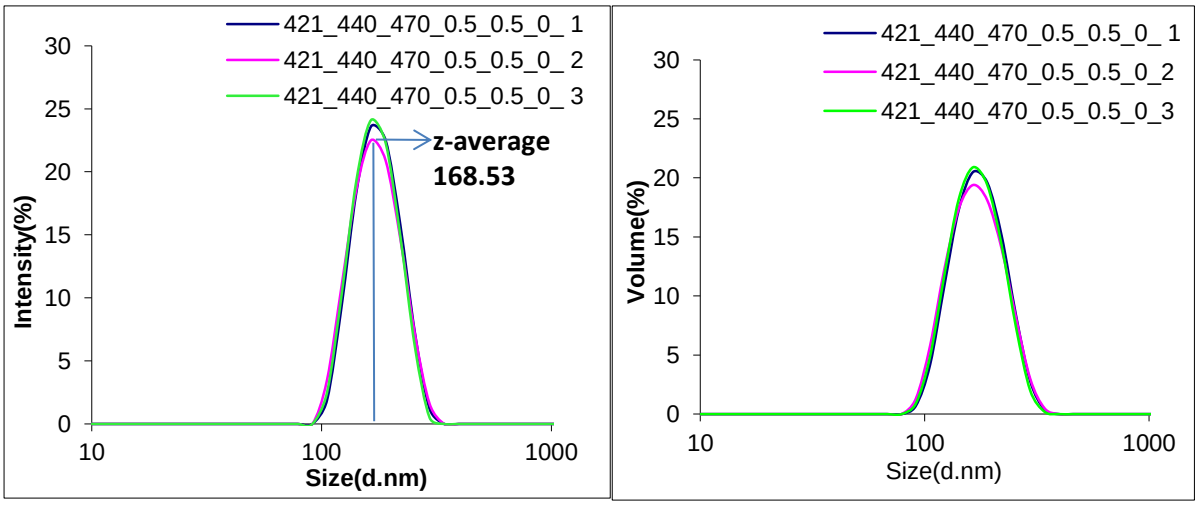


Figure 3.14: Dynamic light scattering measurements of binary mixture of SERS 421 (50%) and 440 (50%) particles. Measurements recorded in triplicate. All necked particles mixture suspended in H_2O.

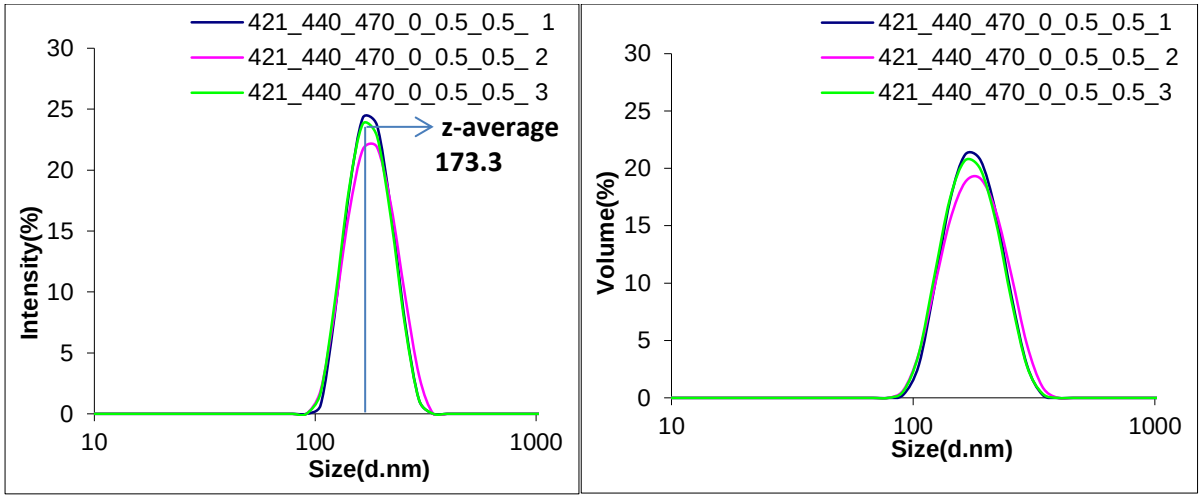


Figure 3.15: Dynamic light scattering measurements of binary mixture of SERS 470 (50%) and 440 (50%) particles. Measurements recorded in triplicate. All necked particles mixture suspended in H_20.

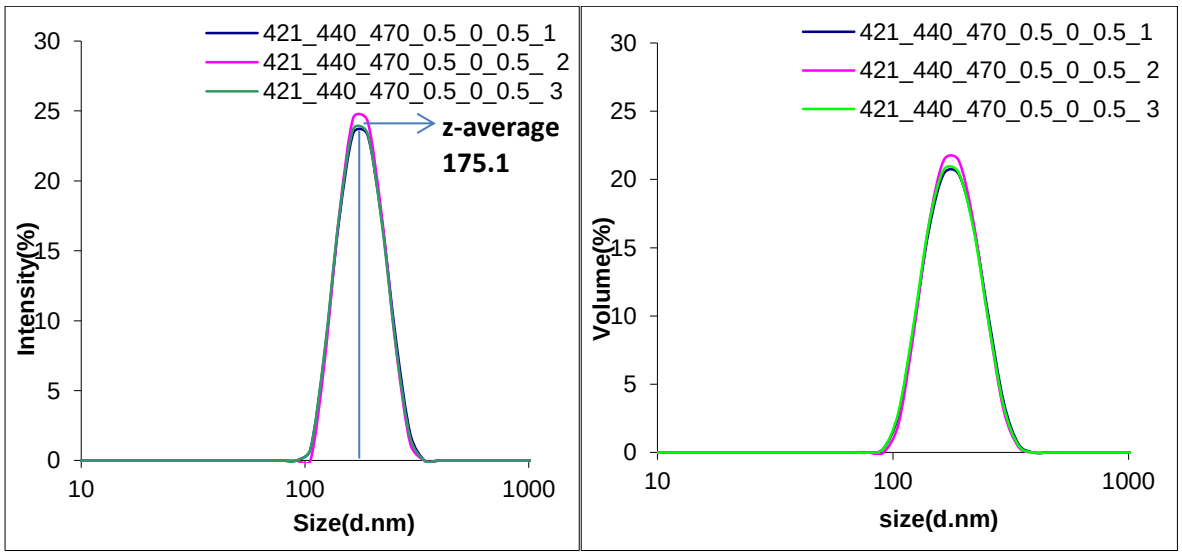


Figure 3.16: Dynamic light scattering measurements of binary mixture of SERS 421 (50%) and 470 (50%) particles. Measurements recorded in triplicate. All necked particles mixture suspended in H_2O.

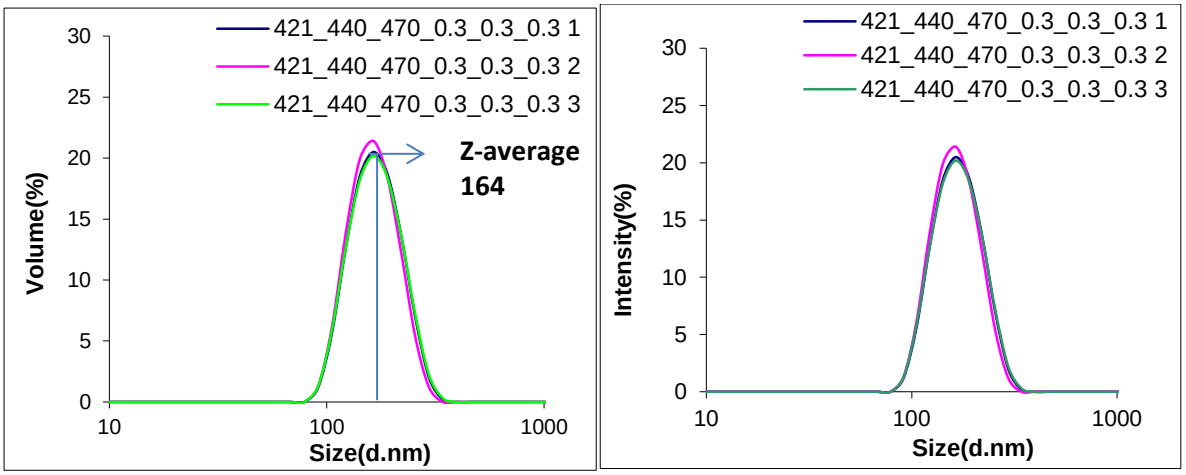


Figure 3.17: Dynamic light scattering measurements of ternary mixture of SERS 421 (33%), SERS 470 (33%) and 440 (33%) particles. Measurements recorded in triplicate. All necked particles mixture suspended in H_2O.

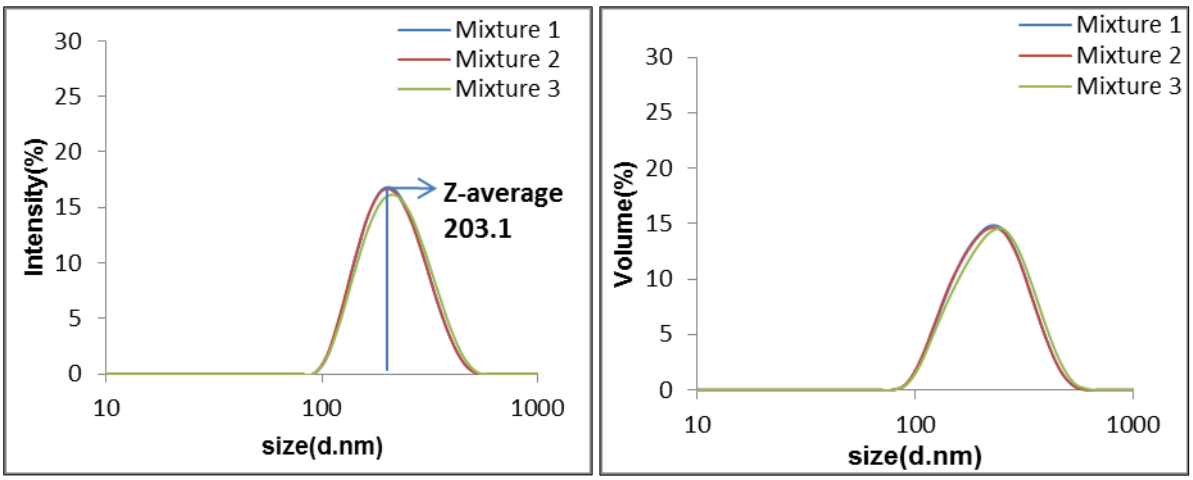


Figure 3.18: Dynamic light scattering measurements of ternary mixture and antibody conjugates (SERS 421-CRP, SERS 470-troponin and 440-myoglobin). Measurements recorded in triplicate. All necked particles mixture suspended in H_2O.

Table 3.2 lists the mean Z-average (size in diameter) and PdI (polydispersity index) values measured for each sample. The data shows that the SERS particles are predominately monodisperse with an average size of 161 nm to 178 nm. The selected mixture is also with the average size of 164-176 nm. The presences of additional peaks were not detected and polydispersity index for each suspension is below 1 that indicating the absence of aggregates in the suspensions.

Sample No	Sample ID	Mean Z-Average (diameter.nm)	Mean PdI (polydispersity index)
1	421	169.7	0.027
2	421 with CRP antibody(conjugated)	186.1	0.007
3	440	167.5	0.017
4	440 with myoglobin antibody(conjugated)	209.1	0.163
5	470	178.7	0.022
6	470 With troponin antibody(conjugated)	206.9	0.114
7	420	161.7	0.016
8	421_440_470(0.5_0.5_0)	168.5	0.013
9	421_440_470(0_0.5_0.5)	173.3	0.011
10	421_440_470(0.5_0_0.5)	175.1	0.013
11	421_440_470(0.3_0.3_0.3)	164.0	0.021
12	Mixture conjugate(470+440+421)	203.1	0.120

Table 3.2: Mean Z-Average and PdI values for the single SERS particles before and after conjugation. Data is an average of three scans.

3.6 Conjugates optimisation

3.6.1 Buffer optimization

In order to identify the most suitable buffer and pH without aggregation troponin conjugates were prepared in borate, phosphate and acetate buffers pH 6.5, 7.2 and 7.5, Figure 3.19 shows the visual identification of aggregation and results are recorded in Table 3.3.

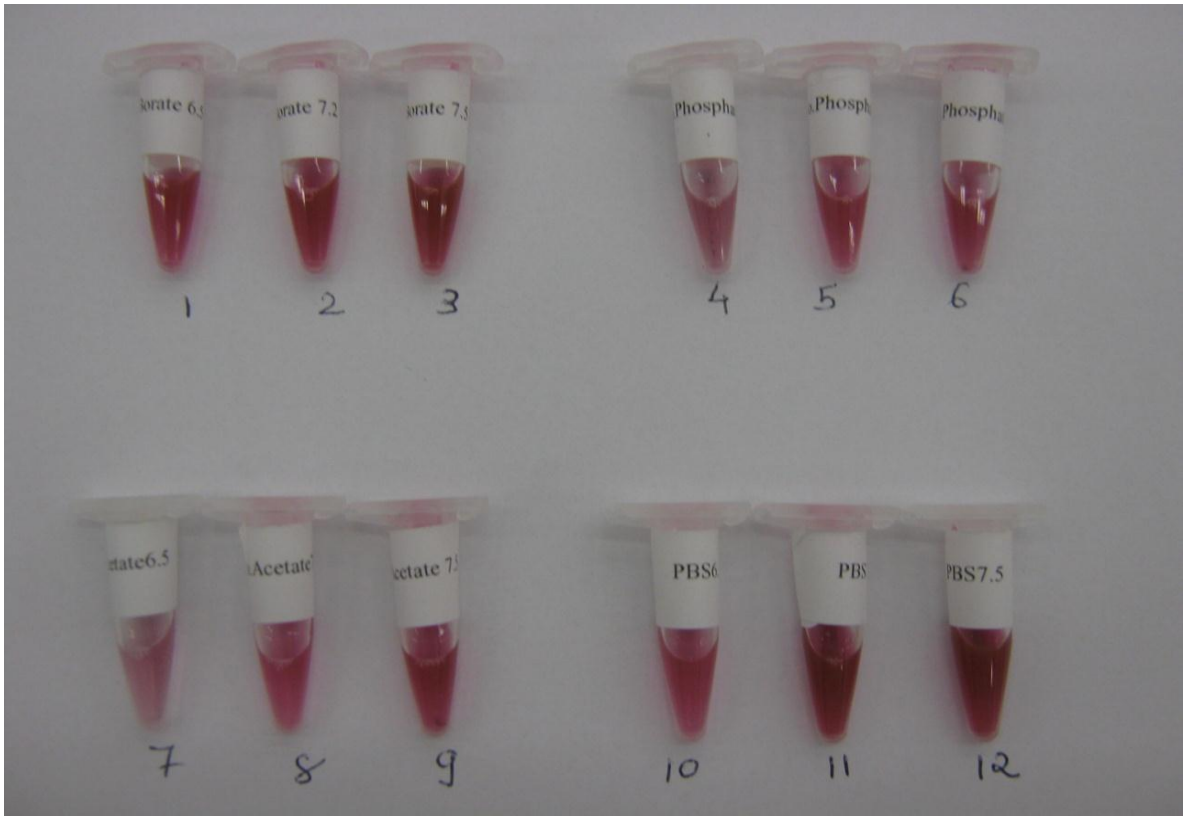


Figure 3.19: Troponin conjugates prepared in four different buffers with different pH values: 6.5, 7.2 and 7.5.
Buffer: (1-3) sodium borate; (4-6) sodium phosphate; (7-9) sodium acetate; and (10-12) phosphate buffered saline. Buffers diluted ten times in relevant storage buffer to visually identify the aggregation, and recorded in Table 3.3. No aggregation observed in 1, 2 and 3, 10 and 11, while in rest of them aggregation observed visually.

Tube no	Conjugate buffer	Visual results
1	sodium borate pH 6.5	no aggregation
2	sodium borate pH 7.2	no aggregation
3	sodium borate pH 7.5	no aggregation
4	sodium phosphate pH 6.5	aggregations seen
5	sodium phosphate pH 7.2	aggregations seen
6	sodium phosphate pH 7.5	aggregations seen
7	sodium acetate pH 6.5	aggregations seen
8	sodium acetate pH 7.2	aggregations seen
9	sodium acetate pH 7.5	aggregations seen
10	PBS pH 6.5	no aggregation
11	PBS pH 7.2	no aggregation
12	PBS pH 7.5	aggregations seen

Table 3.3: Results of troponin conjugate titrations in different buffer and pH.

Each conjugate was also run on a lateral flow strip with troponin (to confirm the aggregation) and without troponin (to see the non-specific binding on the lateral flow strip. Results (Figure 3.20) showed that all conjugates prepared in sodium phosphate, sodium acetate and PBS were aggregating. Conjugates prepared in borate did not show aggregation.

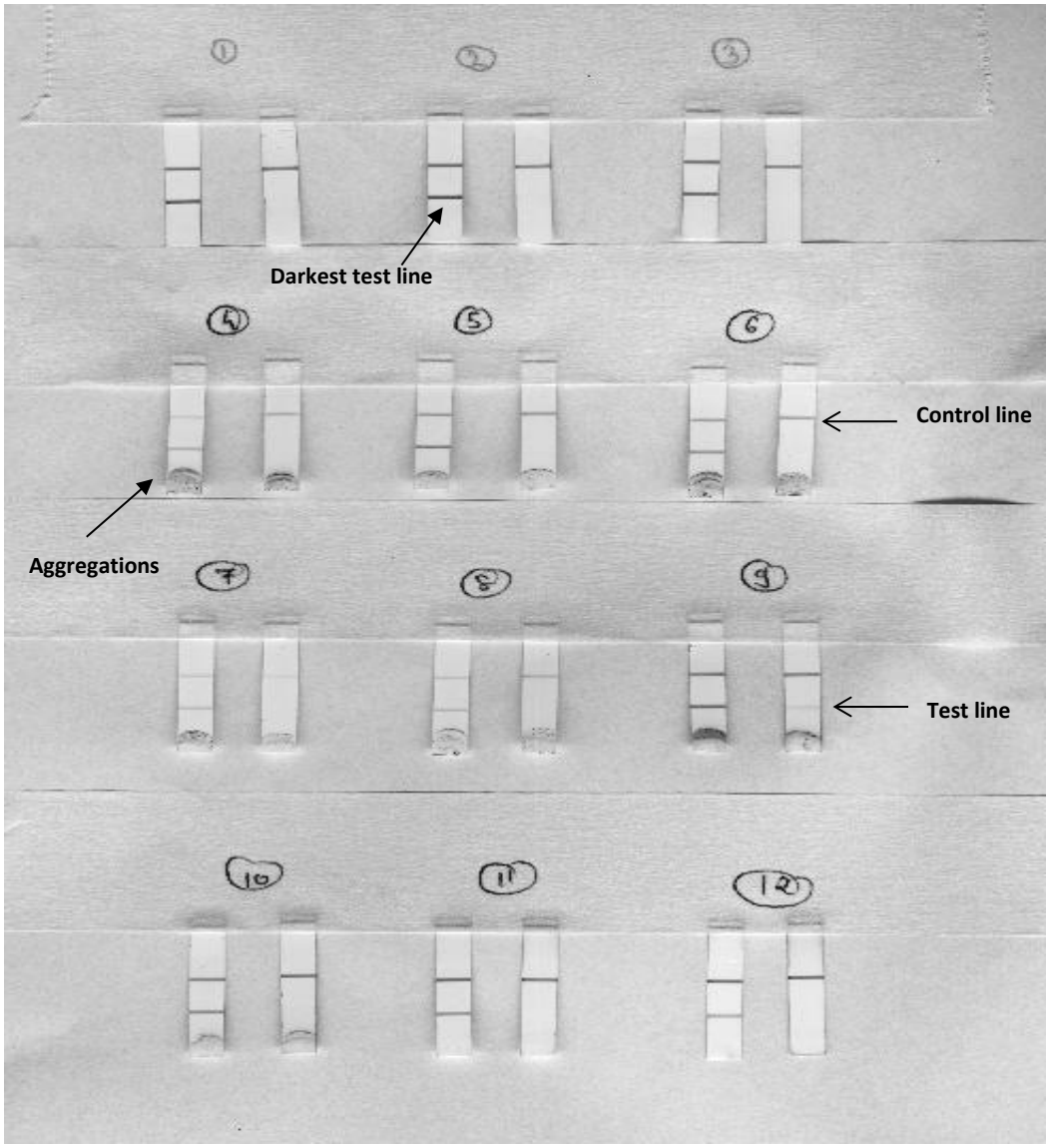


Figure 3.20: Optimisation of the troponin conjugates activity prepared in four different buffers with different pH on a lateral flow strips. No: (1-3) sodium borate 6.5, 7.2 and 7.5; (4-6) sodium phosphate 6.5, 7.2 and 7.5; (7-9) sodium acetate 6.5, 7.2 and 7.5; and (10-12) phosphate buffered saline 6.5, 7.2 and 7.5. No 4 to 10 strips shows aggregation of the particles at starting of the strip. And among all strip no 2 shows the darkest test line.

Likewise troponin titration shown above, myoglobin and CRP titrations were performed in different buffer and pH and most suitable buffers and pH were selected. For troponin and CRP conjugate borate pH 7.2 was the most suitable and for myoglobin borate pH 6.6 was the best.

3.6.2 Antibody: SMCC ratio optimization

Five troponin conjugates with different concentration of antibody - sulfo- SMCC were examined on lateral flow assay (Figure 3.21). In first 3 set conjugate antibody volumes were increased while in set 4 and 5 sulfo-SMCC volumes was decreased (Table 3.4). Troponin strip with 1 mg/ml of (540 + MF4) on test line and rabbit anti-mouse 1mg/ml on control line were used for the testing of each conjugate set. Figure 3.21 shows each sets are having 8 strips where 8 serial dilution of troponin (46, 23.3, 11.6, 5.8, 2.9, 1.5, 0.7, and 0) ng/ml were analysed in CRP free serum and mixed with each set of conjugate (15 µl analyte +1 µl conjugate). Figure 3.21 confirms that set 3 with antibody loading 810 µg/ml gave the best test line colour. While set 1, 2 and 5 were showing aggregations. Particles were crashing at the bottom of the lateral flow strips. Set 4 did not show very strong test line in each strip.

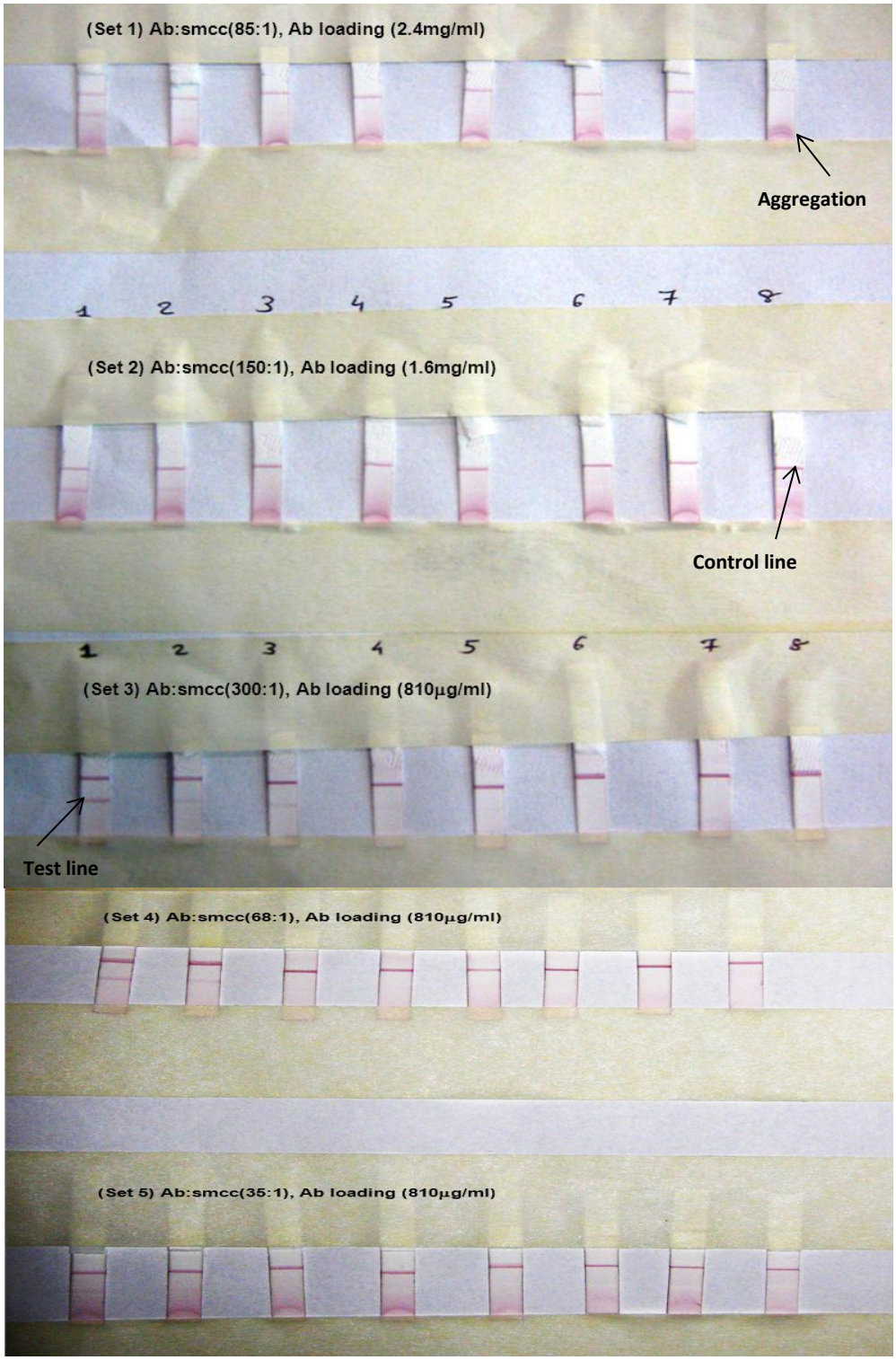


Figure 3.21: Optimisation of the troponin antibody/SMCC ratio by visualisation on lateral flow strips.
[M19C7] mg/ml: (set 1) 2.4; (set 2) 1.6; (set 3 -5) 0.810.
[Antibody/SMCC]: (set 1) 85:1; (set 2) 150:1; (set 3) 300:1; (set 4) 68:1; (set 5) 35:1.
[Troponin] ng/ml: (strip 1) 46; (strip 2) 23.3; (strip 3) 11.61; (strip 4) 5.8; (strip 5) 2.9; (strip 6) 1.5; (strip 7) 0.7; (strip 8) 0.

Detaction Antibody-M19C7 (mg/ml)	Sulfo-SMCC (mg/ml)	Ratio
0.810	2(6.5 µl)	300:01
1.6	2(6.5 µl)	150:01
2.4	2(6.5 µl)	80:01
0.810	2(1.5 µl)	60:01
0.810	2(3.2 µl)	35:01

Table 3.4: Troponin Antibody (M19C7) and sulfo -SMCC concentration Table.

Similarly five myoglobin conjugates were prepared and tested on a strip having myoglobin capture antibody-4E2 (1mg/ml) on test line and rabbit anti-mouse 1mg/ml on control line. Figure 3.22 shows myoglobin conjugate optimisation results where set 4 with detection antibody loading (1.64 mg/ml) shows the darkest test line in all strips. While in sets 1 and 2 did not appear appropriate test line. Set 3 and 5 had aggregation at the bottom of the strip which indicates most of the particles are crashing at the starting point. Table 3.5 shows antibody loading on particles in each sets.

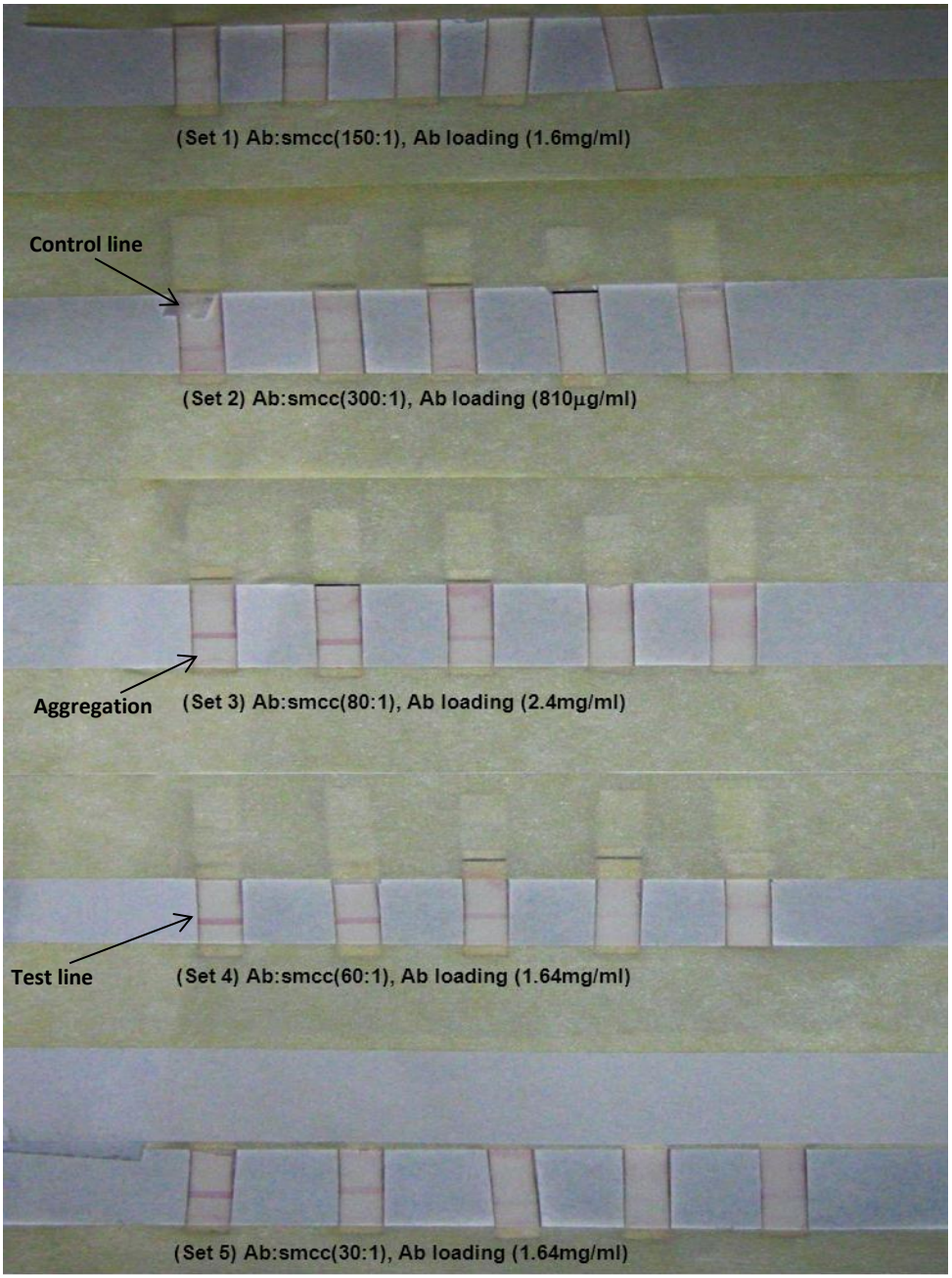


Figure 3.22: Optimisation of the myoglobin antibody/SMCC ratio by visualisation on lateral flow strips.
[7C3] mg/ml: (set 1, 4 and 5) 1.6; (set 2) 0.810; (set 3 -5) 2.4.
[Antibody/SMCC]: (set 1) 150:1; (set 2) 300:1; (set 3) 80:1; (set 4) 60:1; (set 5) 30:1.
[Myoglobin] ng/ml: (strip 1) 500; (strip 2)62.5; (strip 3)7.8; (strip 4) 0.97; (strip 5) 0.

Myoglobin Detaction Antibody-7C3 (mg/ml)	Sulfo-SMCC (mg/ml)	Ratio
1.64	2(6.5 µl)	150:01
0.810	2 (6.5 µl)	300:01
2.4	2(6.5 µl)	80:01
1.64	2(2.6 µl)	60:01
1.64	2(1.5 µl)	35:01

Table 3.5: Myoglobin detection antibody(7C3) and sulfo -SMCC concentration Table.

Figure 3.23 shows 6 strips in 4 sets of conjugates were prepared and tested on a lateral flow strips. Results shows CRP conjugate optimisation results where set 4 with detection antibody loading (1.4 mg/ml) and antibody: SMCC ratio (35:1) showed the darkest test line in all strips. Set 3 had aggregation at the bottom of the strip which indicates most of the particles were crashing at the starting point. Table 3.6 represents antibody loading on particles in each sets and antibody to SMCC ratio.

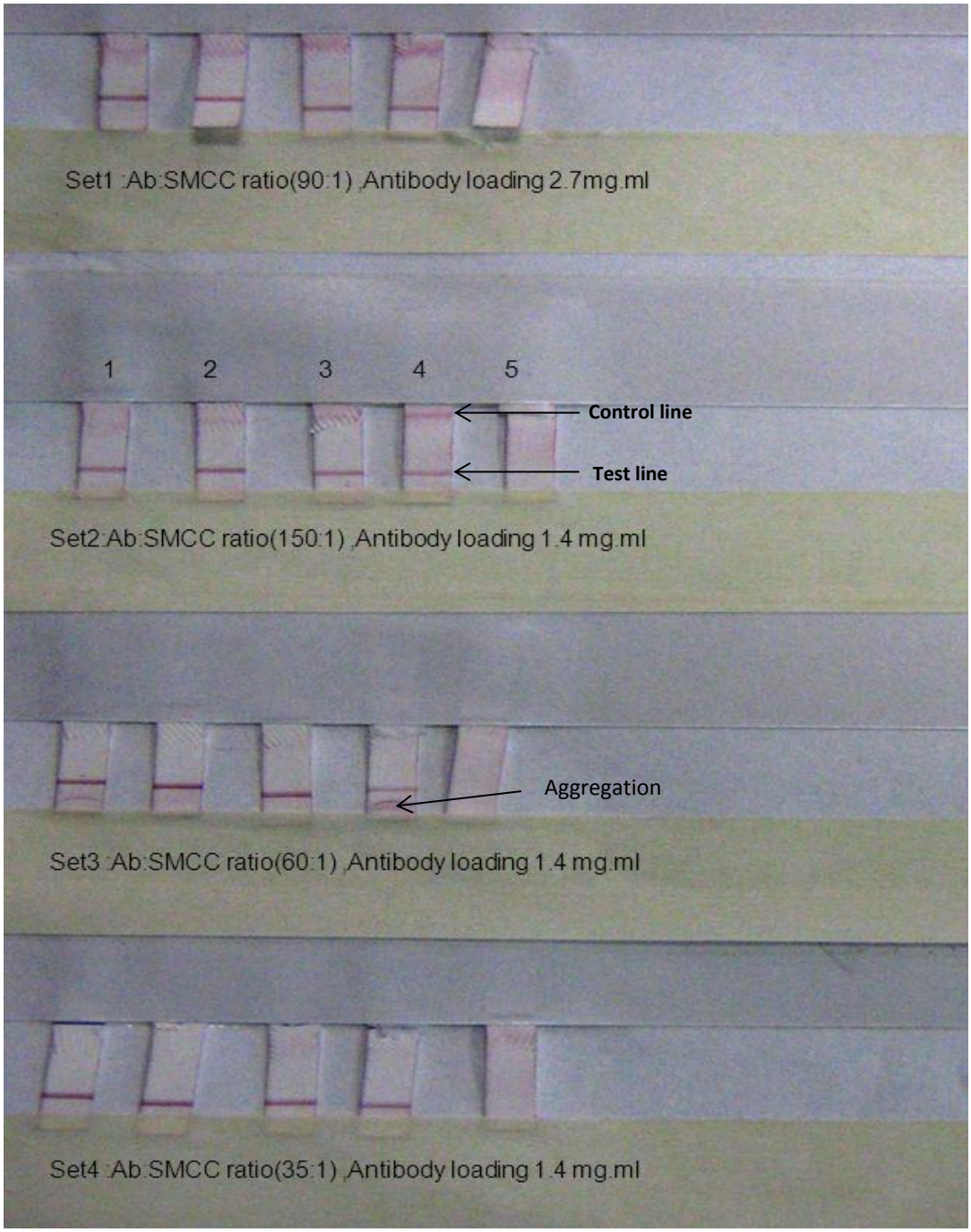


Figure 3.23: Optimisation of the CRP antibody /SMCC ratio by visualisation on lateral flow strips.
[C6] mg/ml: (set 1) 2.7; (set 2, 3 and 4) 1.4.
[Antibody/SMCC]: (set 1) 90:1; (set 2) 150:1; (set 3) 60:1; (set 4) 35:1.
[CRP] ng/ml: (strip 1) 3300; (strip 2) 1100; (strip 3) 370; (strip 4) 110; (strip 5) 0.

CRP Detaction Antibody-C6 (mg/ml)	Sulfo-SMCC (mg/ml)	Ratio
2.7	2 (6.5 µl)	90:01
1.4	2 (6.5 µl)	150:01
1.4	2 (2.6 µl)	60:01
1.4	2 (1.5 µl)	35:01

Table 3.6: CRP antibody(C6) and sulfo-SMCC concentration Table.

3.7 Single analyte lateral flow analysis in buffer

Initially all optimised conjugates were analysed on a single form on lateral flow strips using running buffer to confirm that all conjugates dilutions can be analysed on a lateral flow strip. Graph 3.24 to 3.26 are the results of each conjugates serial dilution strips analysed using the Raman reader and data analysis was performed by combination of Wire and Origin software. Each analyte concentrations are plotted on x-axis and their corresponding read intensity is on Y-axis. Result shows that different concentration of each analyte can detect on lateral flow strip separately. Troponin highest concentration intensity was 80000 RU while CRP highest intensity was 30000 RU. Table 3.7 shows all recorded intensity vs. their concentration.

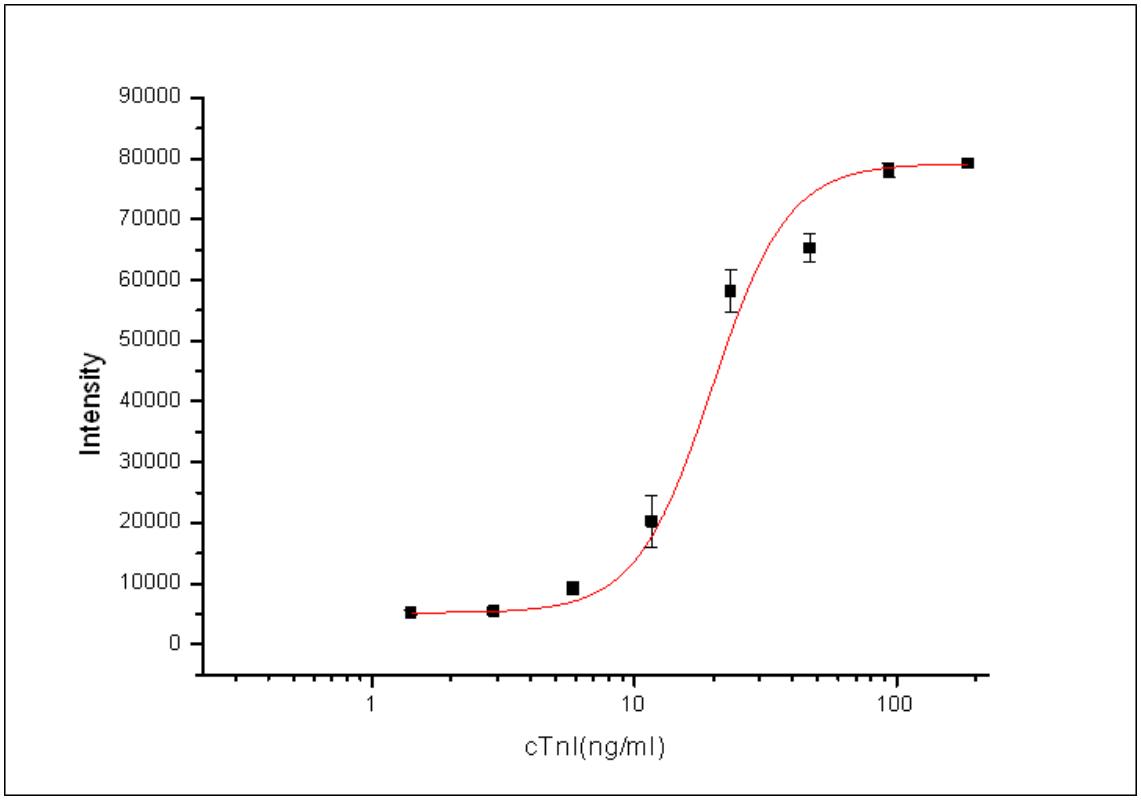


Figure 3.24: Troponin I assay in simplex format on lateral flow strip. Troponin antibody (M19C7) conjugated to SERS 470 particles. Raman intensity read at 1159cm^{-1} using Y-axis scan across the test line. Assay run in running buffer.

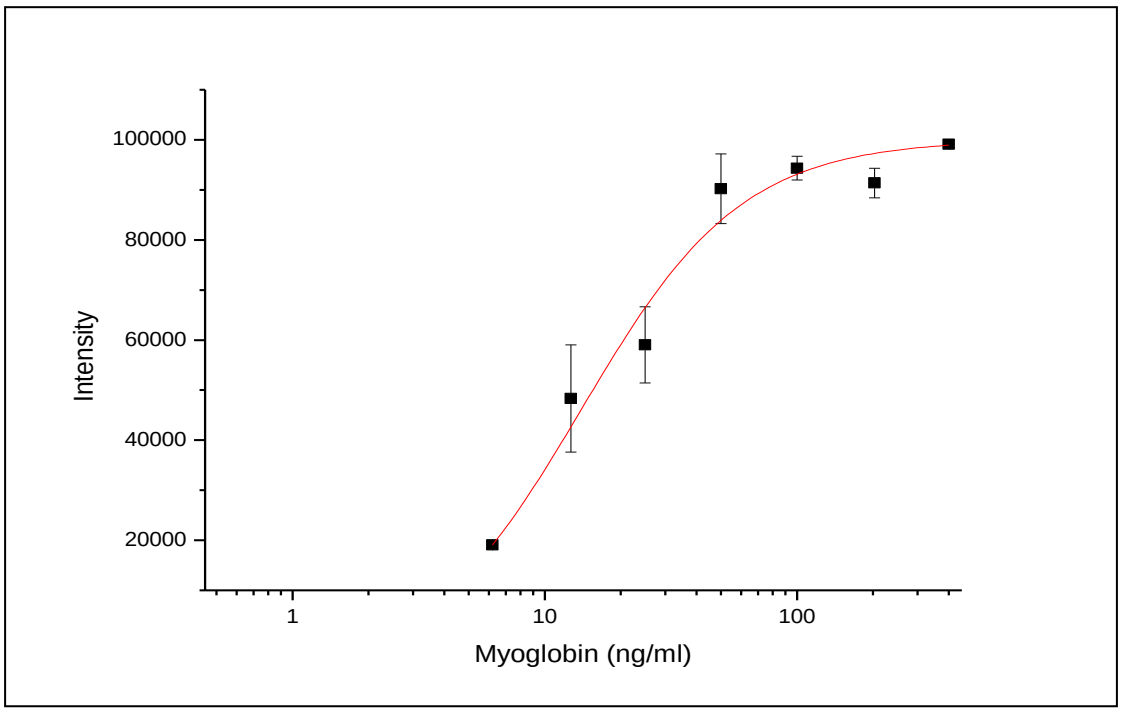


Figure 3.25: Myoglobin assay in simplex format on lateral flow strip. Myoglobin antibody (7C3) conjugated to SERS 440 particles. Raman intensity read at 1335cm^{-1} using Y-axis scan across the test line. Assay run in running buffer.

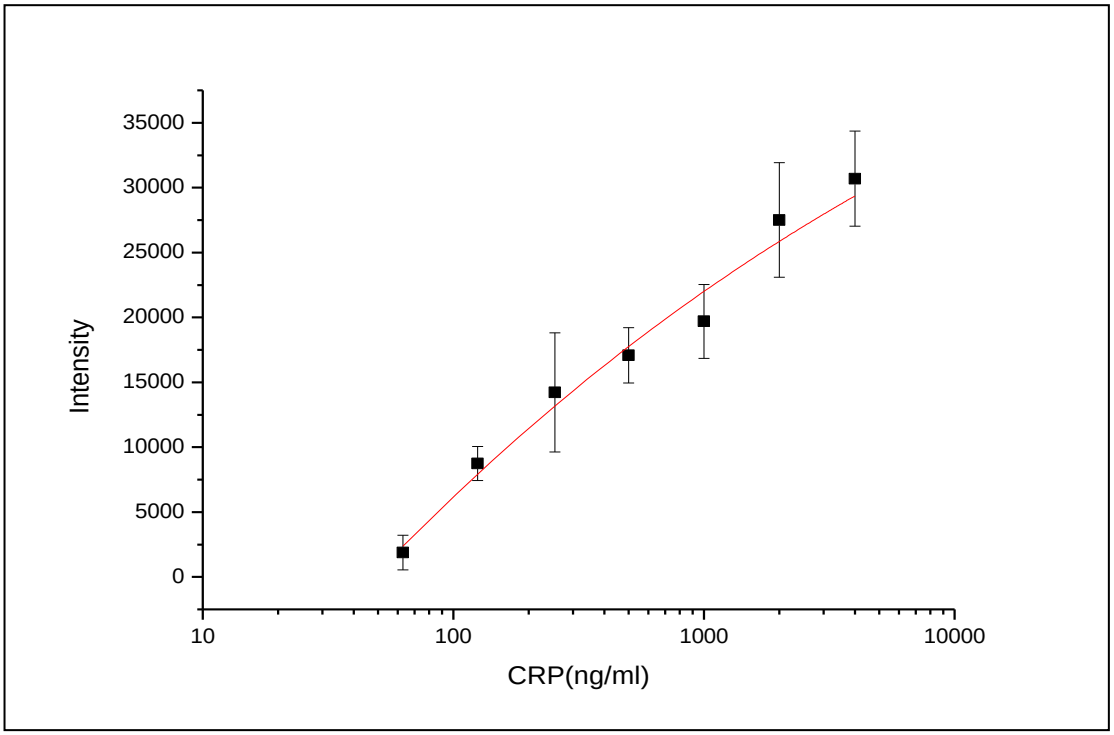


Figure 3.26: CRP assay in simplex format on lateral flow strip. CRP antibody (C6) conjugated to SERS 421 particles. Raman intensity read at 921cm^{-1} using Y-axis scan across the test line. Assay run in running buffer.

Troponin concentration (ng/ml)	Intensity	Myogobin concentration (ng/ml)	Intensity	CRP concentration (ng/ml)	Intensity
1.4	5201	-	-	-	-
2.9	5457	6.2	19048.5	63	1874.5
5.8	9239	12.7	48314.8	125	8742.58
11.6	20214	25	59021.5	254	14222.8
23.3	58136	50	90229.4	500	19903
46.7	65310	100	94341.3	1000	23726.8
93.5	78086	200	91366.7	2000	20644.2
187	79208	400	99135.6	4000	30694.4

Table 3.7: Troponin, myoglobin and CRP concentration vs. Raman intensity recorded on lateral flow strip.

3.8 Single analyte lateral flow analysis in serum

After each conjugate serial dilution analysed in buffer they were analysed in single form on lateral flow strips using serum. Figure 3.27 to 3.29 shows analyte concentration vs. their intensity observed on a Raman reader. For troponin 100 ng/ml to 0.33 ng/ml has been analysed and plotted as figure 3.27. Below Figure 3.28 shows myoglobin can be detected using serum from 412 ng/ml to 3 ng/ml .Similarly CRP can be detected on a lateral flow strip using serum from 4000 ng/ml to 30 ng/ml. Table 3.8 has the verified intensity of their corresponding analyte concentration. Graph shows that all analyte can detect separately using serum.

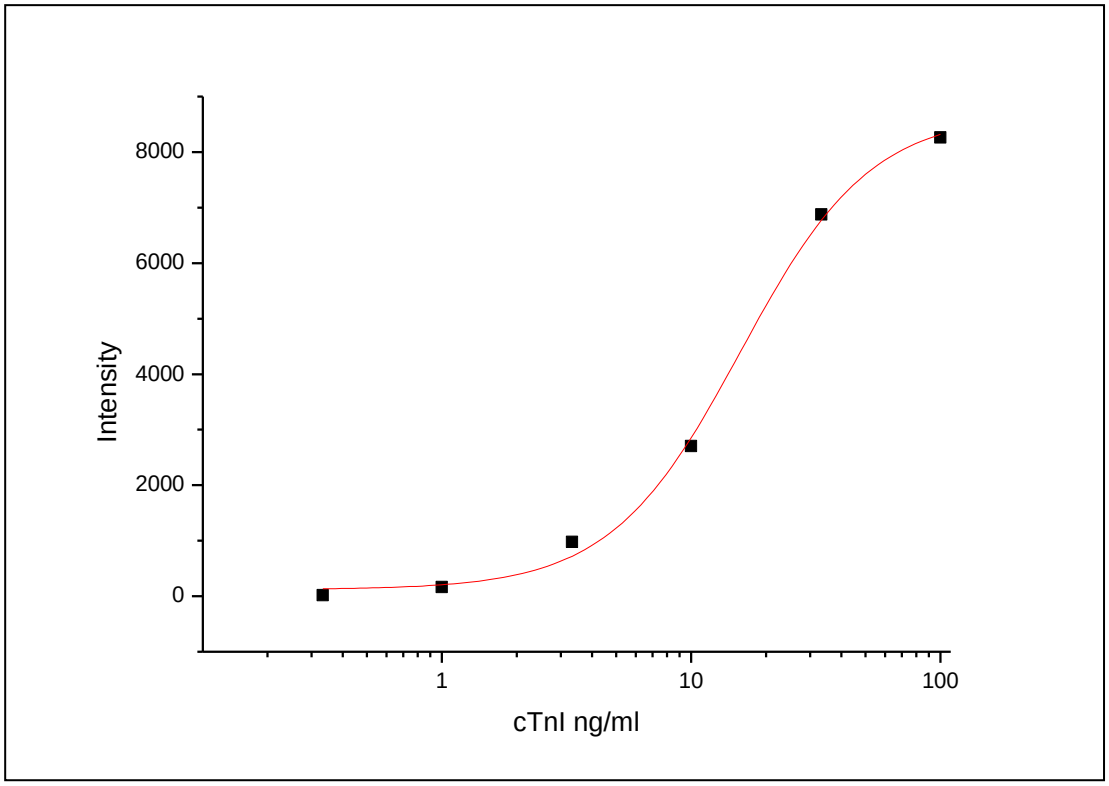


Figure 3.27: Troponin I assay in simplex format on lateral flow strip using serum. Troponin antibody (M19C7) conjugated to SERS 470 particles. Raman intensity read at 1159 cm^{-1} using Y-axis scan across the test line.

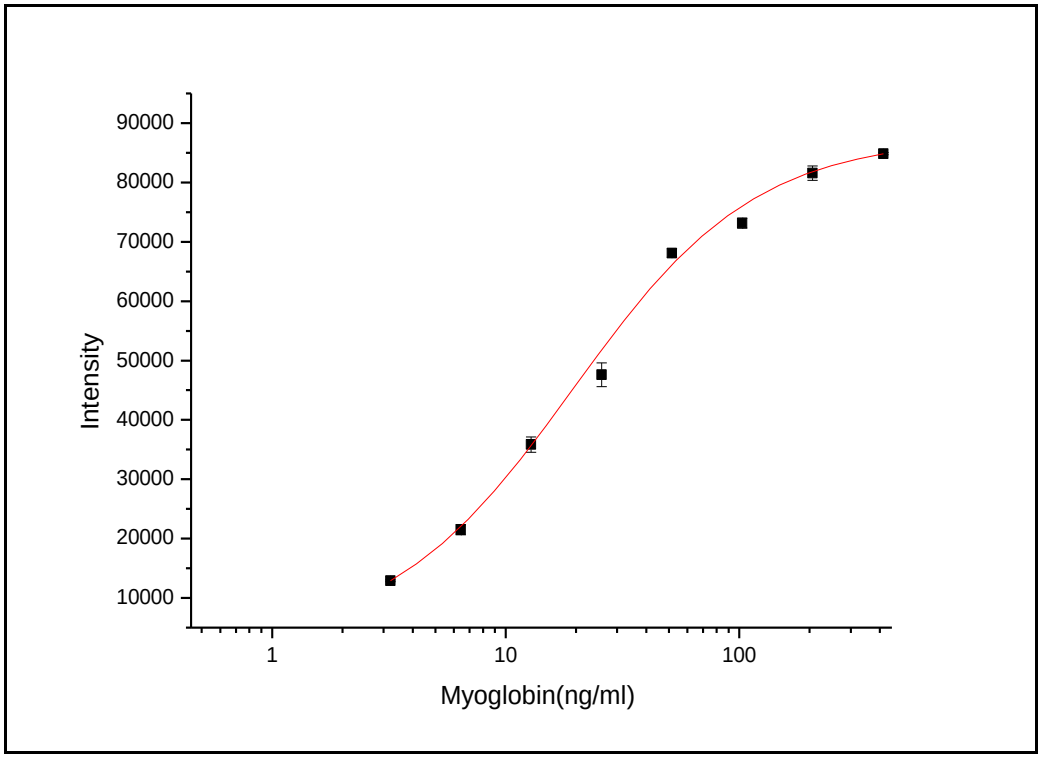


Figure 3.28: Myoglobin assay in simplex format on lateral flow strip using serum. Myoglobin antibody (7C3) conjugated to SERS 440 particles. Raman intensity read at 1335 cm^{-1} using Y-axis scan across the test line.

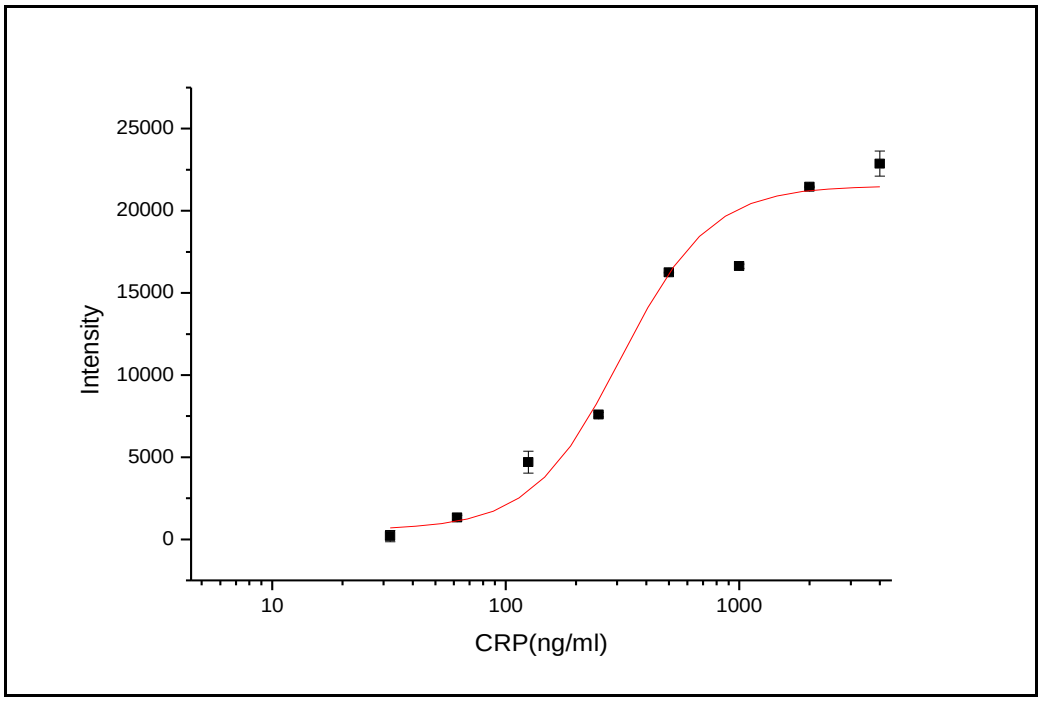


Figure 3.29: CRP assay in simplex format on lateral flow strip using serum. CRP antibody (C6) conjugated to SERS 421 particles. Raman intensity read at 921 cm^{-1} using Y-axis scan across the test line.

Troponin concentration (ng/ml)	Intensity	Myogobin concentration (ng/ml)	Intensity	CRP concentration (ng/ml)	Intensity
0.33	17.6	3.2	12903	32	198
1	163	6.4	21506	64	1335
3.33	977	12.5	35842	125	4698
10	2706	25.7	47605	250	7600
33.3	6877	51.5	68105	500	16240
100	8264	103	73146	1000	16632
		206	81567	2000	21455
		414	84858	4000	22870

Table 3.8: Troponin, myoglobin and CRP concentration vs. Raman intensity recorded on lateral flow strip.

3.9 Discussion

This chapter was laid out a measurement platform for multiplex analysis by investigating various fundamental aspects of the assay using different methodologies. The results of the characterisation and selection of the particles, development of the lateral flow scanning method, preparation and evaluating the conjugates and their application on single lateral flow assay are summarised here.

SERS 420, 440, 470 and 421 particles obtained from Oxonica were studied individually and in mixture using a Raman reader (section 3.1). Examination results of the individual particles identified their unique Raman peaks using the Raman system (R-3000 QE infra-red laser, Ocean Optics, Dunedin, USA). Unique peaks for the 421, 440, 470 and 420 SERS particles were found to be at 921 cm^{-1}, 1335 cm^{-1}, 1159 cm^{-1} and 1077 cm^{-1} respectively. These peaks

were well-separated in the overlaid spectra as well. The result of the mixture analysis by the Raman reader showed that the combination of SERS particles 421, 440 and 470 displayed the most separated Raman peaks. Therefore, this combination of the SERS particles has been chosen for the further conjugation process with troponin, myoglobin and CRP detection antibodies. Calibration of the SERS particles in aqueous suspension verified that the number of particles in the suspension were directly proportional to their observed Raman intensity (section 3.2). Characterisation by UV-Vis absorbance at 543 nm confirmed that the SERS particles were identical in size (section 3.4). Similarly, DLS acquired the size distribution of the particles before and after the antibody loading (section 3.5). DLS analysis established the size of the individual and mixed particles between 161 nm to 178 nm. An increase in each particle's size from 17 nm to 40 nm was observed after loading the antibody on the surface, which is a typical for successfully conjugated particles. No aggregation of SERS particles was observed in individual and mixed form. Thus all characterisation analyses concluded that the SERS particles were suitable for the lateral flow analysis.

Two different scanning methods were evaluated: a three point scan and a y-axis scan. The results revealed that the Y-axis scanning (linear scan) displayed the maximum Raman signals in terms of peak intensity and area (section 3.3). Y-axis scanning method examined the strip with a constant velocity that allowed the collection of maximum data from the SERS particles bound on test line. This method provided more reproducible, stable and fast Raman signals especially for a low concentration of the SERS particles. As a result, all lateral flow assays from this point have been performed using Y-axis scan method.

SERS particle - detection antibody conjugates were titrated in different buffer solutions (sodium borate; sodium phosphate; sodium acetate; and phosphate buffered saline) and pH

conditions (6.5, 7.2 and 7.5). These conjugates were first visually optimised for aggregation and then the lateral flow analysis was performed to confirm the bioactivity by colour development on the test line (section 3.6.1). This investigation identified the following ideal buffer and pH conditions for the preparation of troponin, myoglobin and CRP conjugates: Troponin and CRP conjugates in 50 mM sodium borate pH 7.2 showed darkest test line on the lateral flow strip without any aggregation. While for myoglobin, 50 mM sodium borate pH 6.5 was found to be the most appropriate condition.

Further optimisation of individual antibody: crosslinker (sulfo - SMCC) ratio for troponin, myoglobin and CRP analysis were achieved by visual examination on a lateral flow assay (3.6.2). In order to obtain good quality conjugates for troponin, detection antibody /sulfo-SMCC ratio - 300:1, 150:1, 85:1, 68:1 and 35:1 were analysed and it was found that the 300:1 ratio provided the maximum binding on the test line. For the optimisation of myoglobin, detection antibody / sulfo-SMCC ratio - 300:1, 150:1, 80:1, 60:1 and 30:1 were tested and it was found that the conjugate with the 60:1 ratio gave the maximum bioactivity on the test line. Similarly, visual optimisation of CRP detection antibody / sulfo-SMCC ratio - 150:1, 90:1, 60:1 and 35:1 were examined and the conjugate with 35:1 ratio was found to show the maximum bioactivity on the test line.

470 SERS - troponin conjugate with 300:1 (M19C7: sulfo SMCC) ratio in 50 mM sodium borate pH 7.2, 440 SERS – myoglobin conjugate with 60:1 (7C3: sulfo SMCC) ratio in 50mM sodium borate pH 6.5 and 421 SERS – CRP conjugate with 35:1 (C6: sulfo SMCC) ratio in 50 mM sodium borate pH 7.2 were prepared to obtain single analyte lateral flow assay in buffer and serum. The dose response curve demonstrated that the selected cardiac biomarker can be detected approximately to their dynamic ranges, with a well-separated Raman intensity for each concentration (section 3.7 and 3.8).

In conclusion, this chapter has created a platform for carrying out multiplexed analysis in lateral flow set-up by characterising various materials and optimising the assay conditions.

CHAPTER 4: Multiplexing lateral flow assay

4.0 Introduction

As mentioned in chapter 1 various studies have demonstrated multiplexing by fluorescence and gold nanorods. However, quenching of signals or multiple steps for the detection were the major issues in achieving a quantitative assay (Huang *et al*., 2012; Worsley *et al*., 2012). To overcome these issues this chapter aims to demonstrate the potential of SERS particles in the analysis of multiple analytes in a single step.

The SERS multiplex detection technique was designed as a sandwich assay in which the target analyte was bound in between SERS particles labelled with a "detection" antibody and a "capture antibody" on the test line. The ratio of signals emitted by the antibodies labelled SERS particles revealed the analyte concentration directly in the complex mixture.

The best combination of the SERS particles and its evaluation with a cardiac biomarker antibody on a simplex lateral flow assay using buffer and serum was achieved in the previous chapter. The calibration and characterisation analyses have quantified the SERS particle and antibody attributes and quantity to be used in the mixture assay. Now, this chapter's focus was to develop a multiplex assay for the chosen cardiac biomarkers on a single test line using the optimised conditions identified previously.

In order to accomplish the target of multiplex detection the following was planned in this chapter: (1) Initial multiplexing measurements in serum for all three analytes using optimised parameters identified from the simplex lateral flow assay; (2) Refinement of multiplexing assay in serum; (3) Effect of high, medium and low concentration of analytes in multiplexing and (4) Investigation of interference in multiplexing assay. These are now described in detail below.

(1) Initial multiplexing assay on lateral flow strip in serum.

This preliminary examination was intended to ascertain the behaviour of each biomarker in a mixture. It also aimed to identify the essential modifications required to acquire well separated Raman peak for each analyte in the final multiplex assay. In the designed model all capture antibody (MF4+560, 4E2 and C2) were loaded in a mixture on 1 mm test line and the best conditions for the preparation of conjugates identified in the previous chapter were used to for the initial demonstration of the multiplex assay in serum. Equal amounts of conjugates (troponin, myoglobin and CRP) were loaded on the lateral flow strip. All the conditions of the multiplex assay were kept same as the simplex assay to make the Raman signal intensity obtained in the mixture comparable with the previously obtained individual measurement outputs (section 4.1).

(2) Refinement of multiplexing assay in serum.

Antibody labelled SERS particles may behave differently in a mixture compared to when tested individually. Consequently, this segment was designed to improve the assay conditions in mixture until acceptable distinct Raman signals for each sample dilution were obtained. Based on the section 4.1 outcomes additional multiplexing assay was conducted with increase in particles-detection antibody loading (increased myoglobin and troponin conjugate). From the mixture analysis, individual dose-response curves for troponin, myoglobin and CRP were obtained according to their individual pathological range in an immunoassay (section 4.2).

(3) Effect of high, medium and low concentration of analytes in multiplexing assay.

In a disease state, the serum or blood sample may have elevated or negligible biomarkers in a blend form (Kelley *et al.,* 2009; McDonnell *et al*., 2009) and hence this could affect the measurements of the targeted biomarker measurements. With this purpose in mind

artificial condition were created in serum by adding diverse combination of the biomarkers. For example, the effect of a high CRP concentration (1800 ng/ml) with low troponin concentration (21 ng/ml) on medium myoglobin concentration (200 ng/ml) was investigated. Twenty seven different combinations of biomarkers were tested (section 4.3).

(4) Investigation of interference in multiplexing assay:

The aim of this section was to inspect interference concerns to obtain a qualitative and quantitative assay. Thus, following on from the results of section 4.3 this section investigated the interference issues in a targeted analyte Raman intensity by discretely adding each co-analyte. The examinations were carried out to find out the most effected biomarker and the level of interferences. As a result, section 4.4 has examined the following: (1) myoglobin and CRP interference on troponin intensity, (2) Troponin and CRP interference on myoglobin intensity and (3) troponin and myoglobin interference on CRP intensity.

4.1 Initial multiplexing assay on lateral flow strip in serum

An analyte mixture (troponin, myoglobin and CRP) was serially diluted and tested on a lateral flow strips. Figure 4.1 to 4.3 and Table 4.1 represent the individual candidate's measured Raman intensity against the corresponding concentration. In all three graphs at each concentration other 2 candidates are present. In simple term all analyte intensity were deconvoluted and separately plotted in below to understand the assay. Total 7 concentrations in serial dilution were measured for each candidate and intensity is represented in Table 4.1. Compare to single analyte assay from chapter 3 (Figure 3.27) troponin shows dissimilar behaviour when it is in mixture form. When it was in single system troponin intensity was almost double for most of the concentrations and each concentration intensity values were well separated. Figure 4.1 shows that the troponin intensity is very poor in multiplexing form. It gives total intensity increase only by 2000 RU from lowest (0ng/ml) to highest concentration (65.5ng/ml). Therefore it concludes that further assay needs to be done by addition of more troponin conjugate or analyte in the assay which may give improvement in the intensity. Similarly compare to Figure 3.28 myoglobin intensity from multiplex assay (Figure 4.2) is ten times lower. Higher concentration (400ng/ml) of myoglobin had the intensity of 15000 RU in presence of other 2 analyte in high concentration. Figure 4.3 shows CRP behaviour was similar as single analyte (Figure 3.29). These results clearly show that myoglobin and troponin assay intensity needs improvement in multiplexing form.

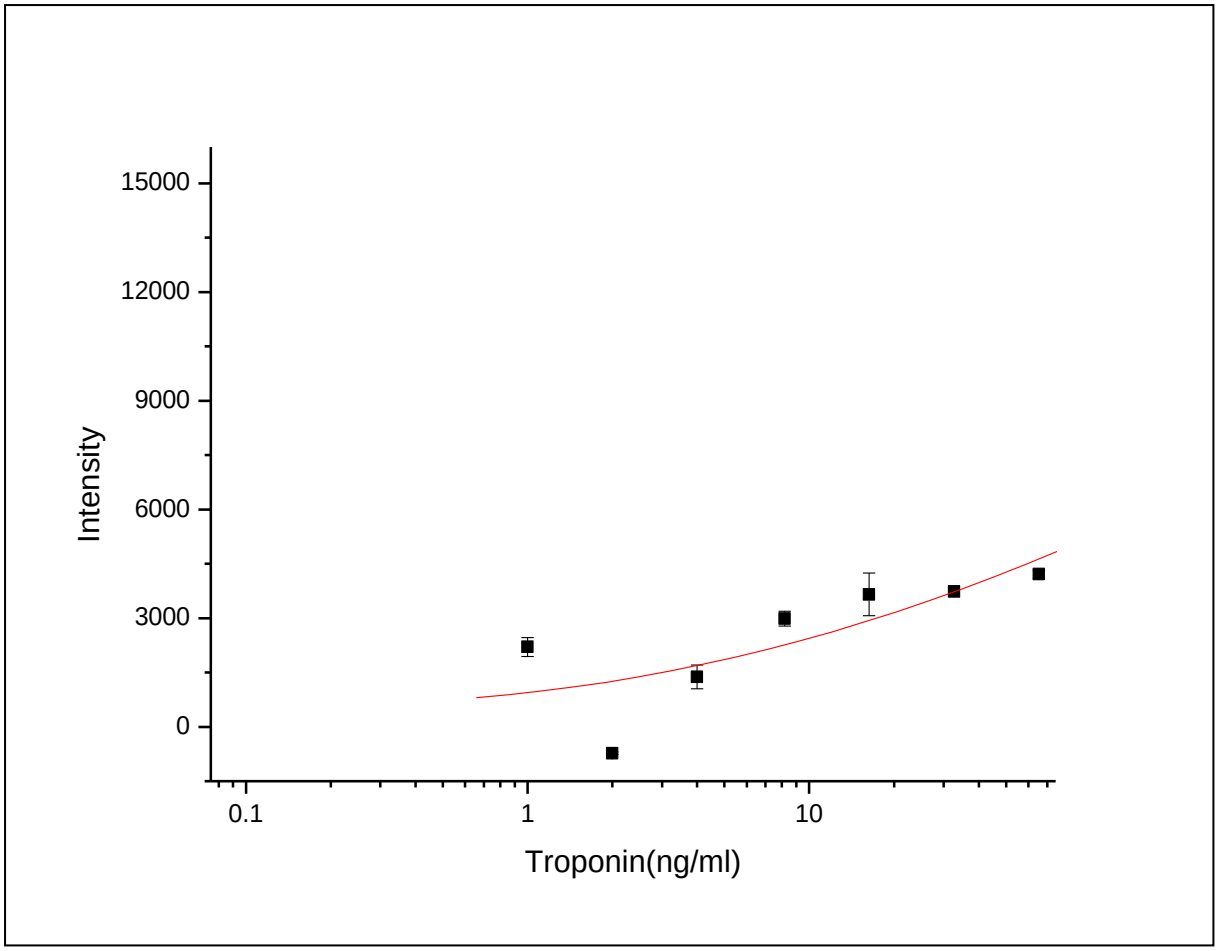


Figure 4.1: Calibration of troponin assay in serum with multiplex format (using optimised simplex parameters). Troponin intensity read at 1159 cm^{-1} using Y-axis scan across the test line. Optimised simplex parameters:
[Myoglobin]: 400-6 ng/ml; [CRP]: 4000-60 ng/ml.
[Myoglobin conjugate]: 0.3 µl/strip; [CRP conjugate]: 0.3 µl/strip.
Vertical bars indicate +/- standard deviation (n=3).

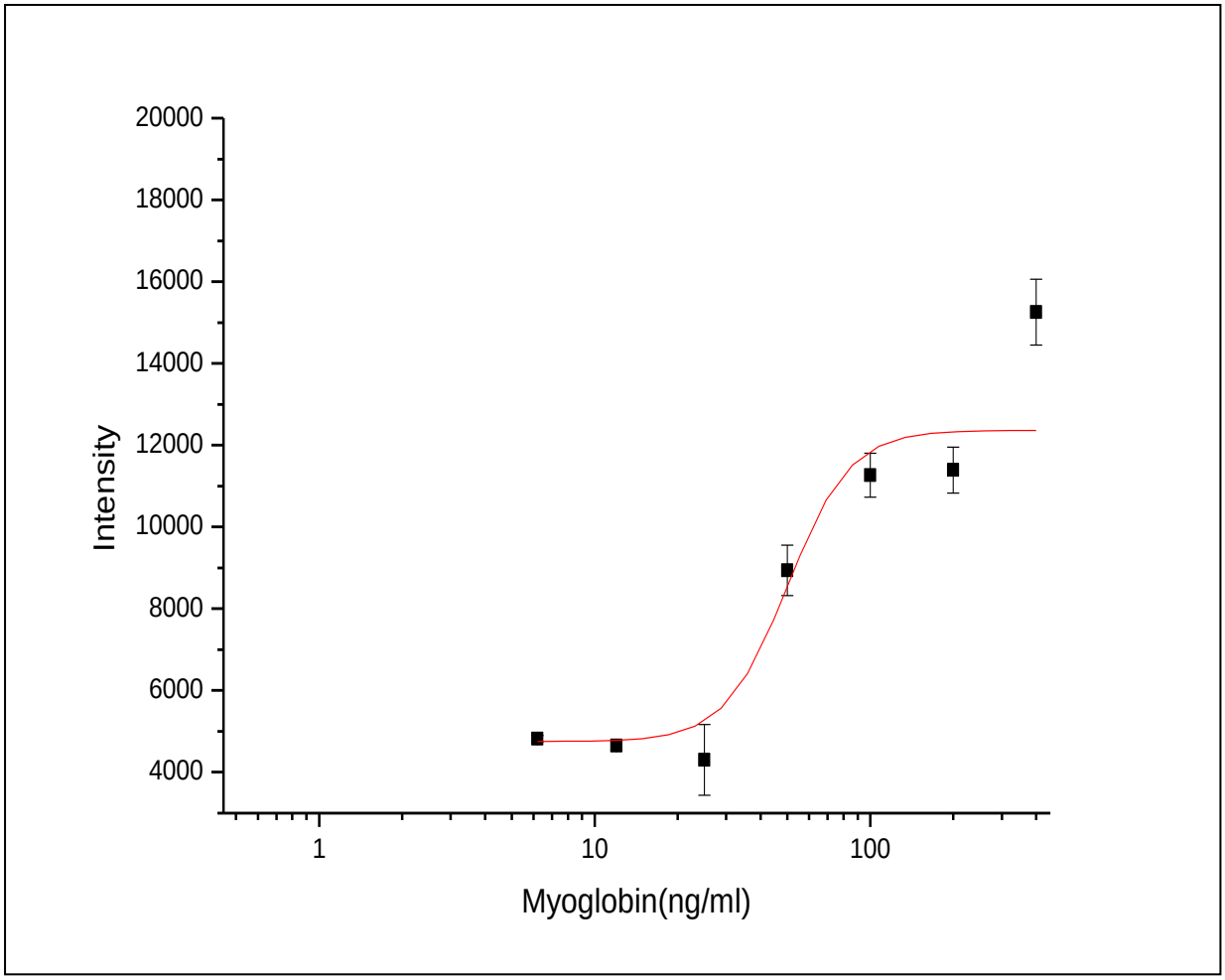


Figure 4.2: Calibration of myoglobin assay in serum with multiplex format (using optimised simplex parameters). Myoglobin intensity read at 1335 cm^{-1} using Y-axis scan across the test line. Optimised simplex parameters:
[Troponin]: 66-1 ng/ml; [CRP]: 4000-60 ng/ml.
[Troponin conjugate]: 0.3 µl/strip; [CRP conjugate]: 0.3 µl/strip.
Vertical bars indicate +/- standard deviation (n=3).

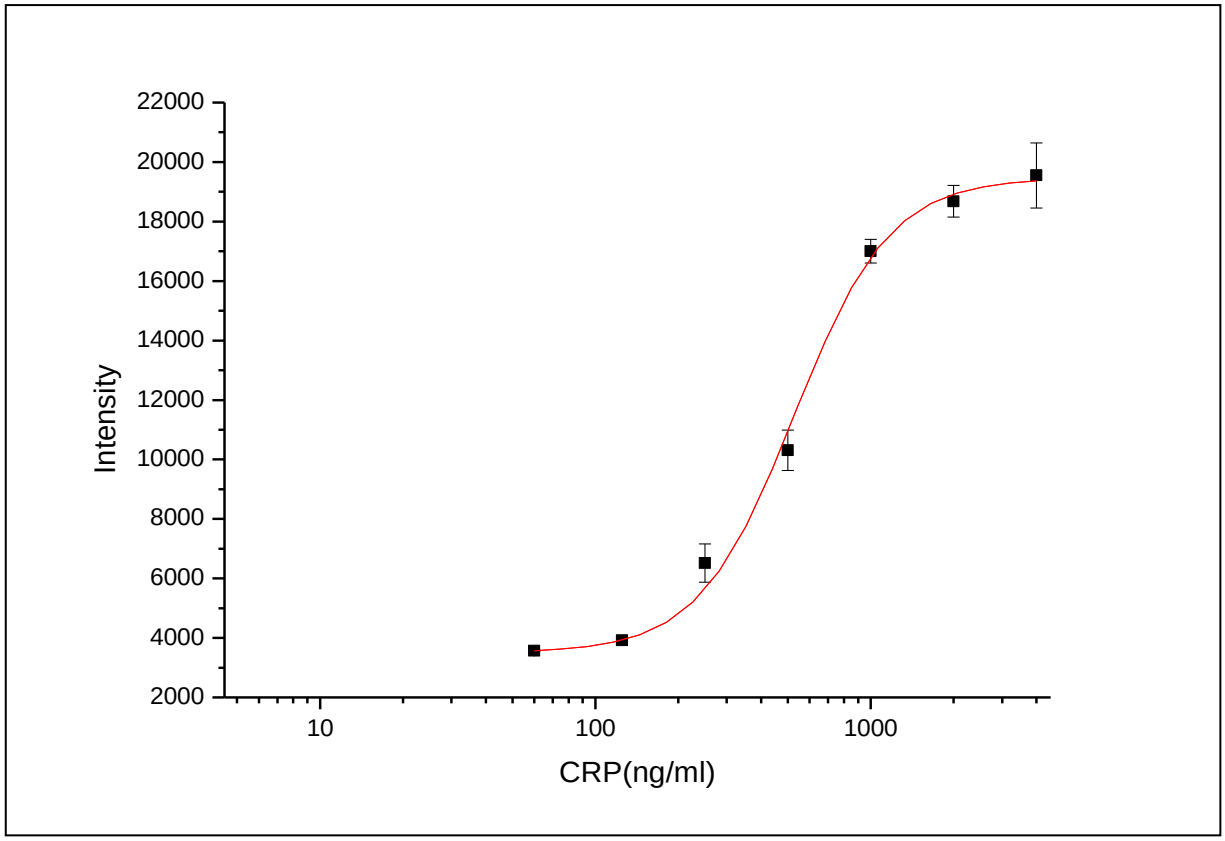


Figure 4.3: Calibration of CRP assay in serum with multiplexing format (using optimised simplex parameters). CRP intensity read at 921 cm^{-1} using Y-axis scan across the test line. Optimised simplex parameters:
[Troponin]: 66-1 ng/ml; [Myoglobin]: 400-6 ng/ml.
[Troponin conjugate]:0.3 µl/strip; [Myoglobin conjugate]: 0.3µl/strip.
Vertical bars indicate +/- standard deviation (n=3).

Strip No	CRP (ng/ml)	Intensity	Troponin (ng/ml)	Intensity	Myoglobin (ng/ml)	Intensity
1	4000	19551	65.5	4210	400	15253
2	2000	18680	32.7	3735	200	11391
3	1000	17001	16.3	3655	100	11266
4	500	10309	8.18	2988	50	8937
5	250	6517	4	1376	25	4304
6	125	3917	2	-729	12	4655
7	60	3572	1	2203	6	4818

Table 4.1: CRP, myoglobin and troponin concentration in each multiplex strip and corresponding intensity from Raman reader.

In summary, initial multiplexing results were obtained using antibody loading that were appropriate in a simplex format but the troponin intensity was very low compare to

myoglobin and CRP assay. In addition, the 'quality' of the troponin and myoglobin results was poor.

4.2 Multiplexing assay in serum

In an attempt to improve the multiplexed troponin and myoglobin assays, the loadings of troponin and myoglobin conjugates were increased from previous experiment. Sample loading method is mention in chapter 2 section 2.5. Here Figure 4.4 to 4.6 represents the results for this assay.

Associate to previous multiplexing attempt (section 4.1) troponin intensities are enhanced four times higher and each concentration gives linear response. These results displays that by increasing conjugate loading of myoglobin and troponin in the assay, it improves their intensity as well. All measured concentration of analyte and their Raman intensity are recorded in Table 4.2.

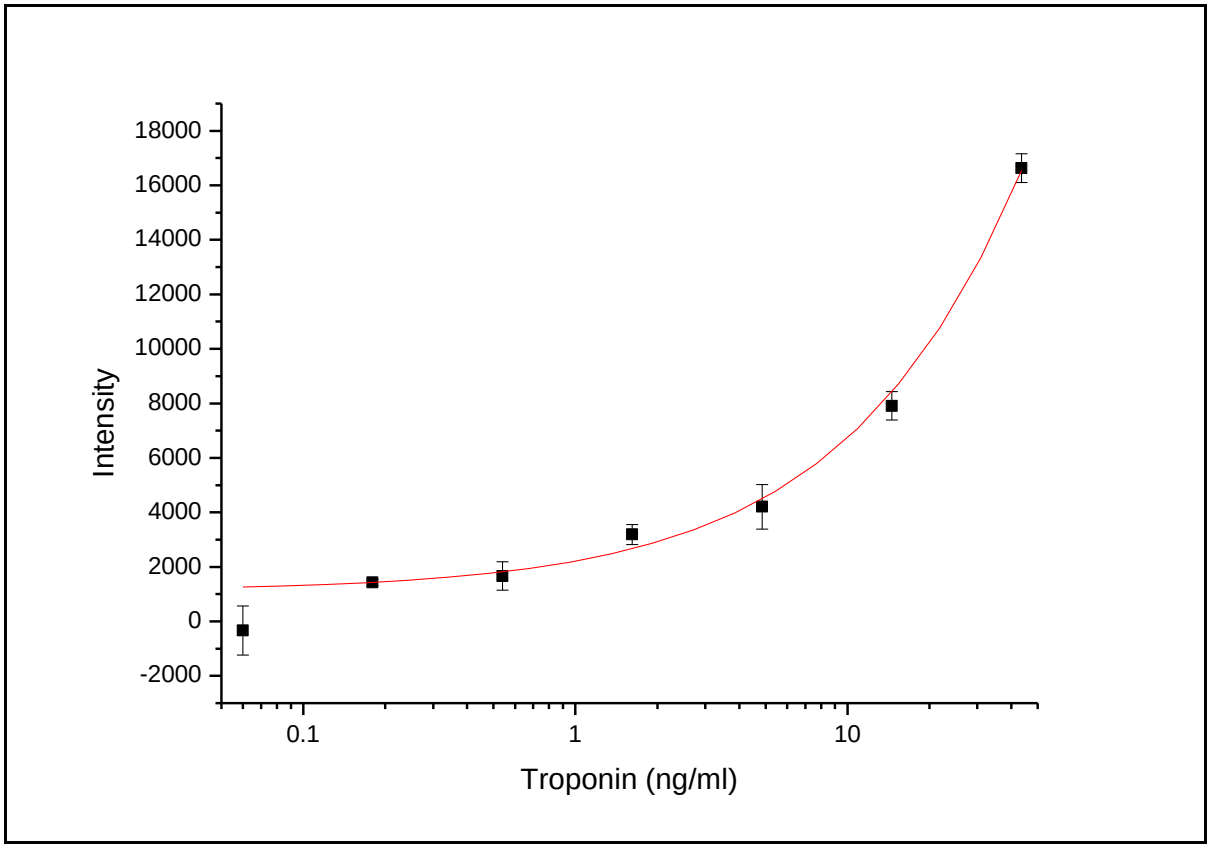


Figure 4.4: Calibration of troponin assay in serum with multiplex format (using optimised simplex parameters). Troponin intensity read at 1159 cm^{-1} using Y-axis scan across the test line. Optimised simplex parameters:
[Myoglobin]: 533-0.7 ng/ml; [CRP]: 5333-7 ng/ml.
[Myoglobin conjugate]: 0.4 µl/strip; [CRP conjugate]: 0.3 µl/strip.
Vertical bars indicate +/- standard deviation (n=3).

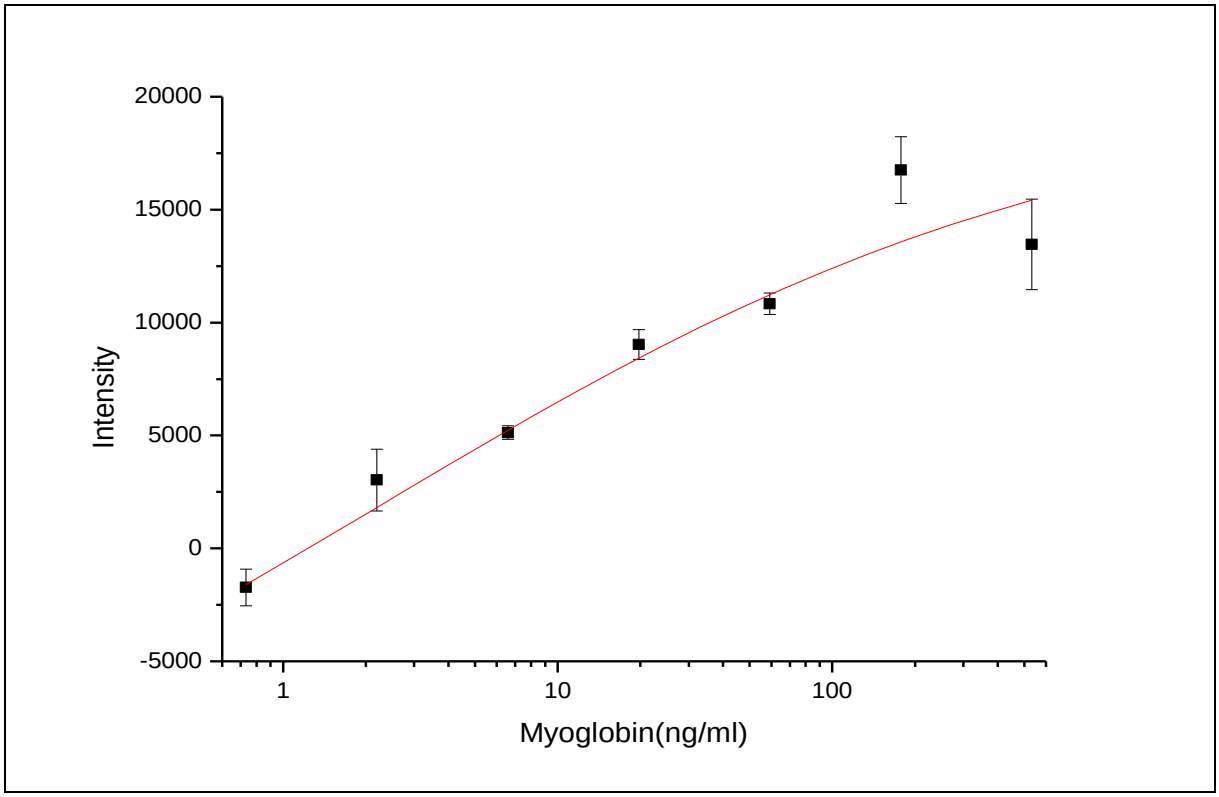


Figure 4.5: Calibration of myoglobin assay in serum with multiplex format (using optimised simplex parameters). Myoglobin intensity read at 1335 cm^{-1} using Y-axis scan across the test line. Optimised simplex parameters:
[Troponin]: 44-0.06 ng/ml; [CRP]: 5333-7 ng/ml.
[Troponin conjugate]: 0.4 µl/strip; [CRP conjugate]: 0.3 µl/strip.
Vertical bars indicate +/- standard deviation (n=3).

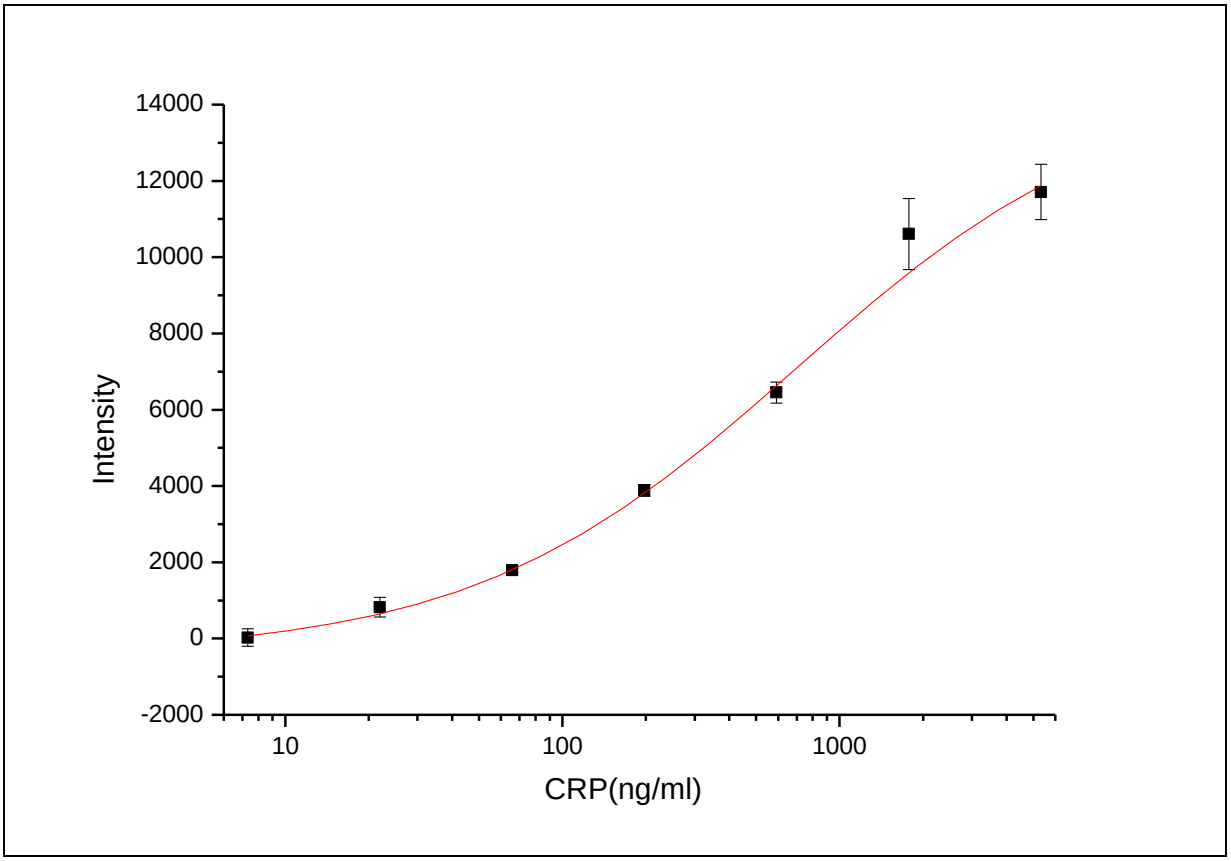


Figure 4.6: Calibration of CRP assay in serum with multiplex format (using optimised simplex parameters). CRP intensity read at 921 cm^{-1} using Y-axis scan across the test line. Optimised simplex parameters:
[Troponin]: 44-0.06 ng/ml; [Myoglobin]: 533-0.7 ng/ml.
[Troponin conjugate]: 0.4 µl/strip; [Myoglobin conjugate]: 0.4 µl/strip.
Vertical bars indicate +/- standard deviation (n=3).

Strip No	CRP (ng/ml)	Intensity	Troponin (ng/ml)	Intensity	Myoglobin (ng/ml)	Intensity
1	5333	11710	43.7	16636	533	13459
2	1778	10608	14.6	7909	178	16751
3	593	6449	4.9	4203	59	10835
4	198	3887	1.6	3190	20	9031
5	66	1789	0.6	1666	7	5129
6	22	822	0.2	1432	2.2	3030
7	7	26	0.06	-339	0.7	-1726

Table 4.2: CRP, myoglobin and troponin concentration in each multiplexing strip and corresponding intensity read on Raman reader.

Multiplexing assay results shows that all three analyte are separately detectable on multiplex format on a single test line of lateral flow strip and their detection range are as

below in presence of other two analyte. CRP was detected from 5 ng/ml to 6000 ng/ml; troponin was detected from 0.1 ng/ml to 50 ng/ml and myoglobin can be detected from 0.5 ng/ml to 600 ng/ml.

4.3 Effect of high, medium and low concentration of analytes in multiplexing

Multiplexing results in section 4.2 showed the detection of all three analytes but it did not explain that presence of one analyte in higher or low concentration changing the intensity of detection analyte at fix concentration intensity. Therefore, the aim of this work was to investigate whether or not the addition of the other two analyte in various concentrations would change the analytical signals. CRP, troponin and myoglobin standard curves (Figure 4.4 to 4.6) were used as a reference and plotted as a red line in below with the concentration (■) in graphs (4.7 to 4.9). Three (High, Medium and low) concentration were chosen for the examination. Figure 4.7 at selected 3 concentration of troponin (50, 5, 0.4 ng/ml) when other 2 candidate (CRP and myoglobin) are present in high concentration (1800, 178 ng/ml) the troponin intensity is similar to previous assay and represented as (♦). When only CRP is loaded in low concentration ((21 ng/ml) and myoglobin was kept high ((178 ng/ml), that has affected the troponin intensity as well. That is represented as (♦) in the graph. Similarly when CRP was kept content as high (1800 ng/ml) and alter myoglobin concentration as low (6.5 ng/ml), it again influence in the troponin intensity .that is represented as (♦). When both of these candidates (CRP and myoglobin) are present in lower concentration (21, 6.5 ng/ml) troponin intensity was increased (♦). Here in all graphs only high and low effect is plotted to avoid more complexity .Analogous to troponin graph other 2 analyte Figure 4.5 and 4.6 shows the changes in the selected analyte intensity when there is a small alteration in the concentration of other candidates present in sample

mixture. Each strips and their corresponding concentration of three analytes are represented in chapter 2 section 2.11.

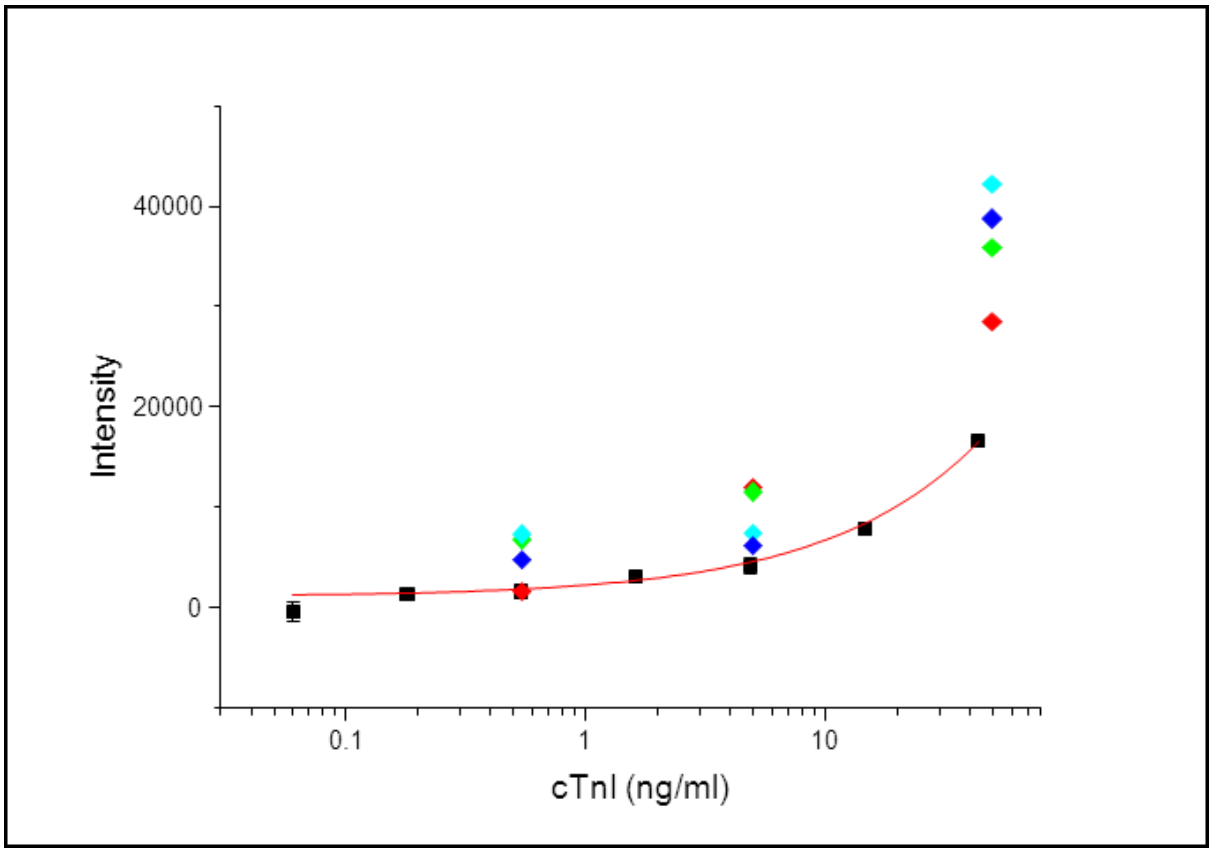


Figure 4.7: Effect of myoglobin and CRP on troponin assay at high, medium and low concentration. Troponin intensity read at 1159 cm^{-1} using Y-axis scan across the test line. Assay run in serum.
[CRP] ng/ml: 1800 (♦); 1800 (♦); 21 (♦); 21 (♦) and 0 (■).
[Myoglobin] ng/ml: 178 (♦); 6.5 (♦); 178(♦); 6.5 (♦) and 0 (■).
[Troponin conjugate]: 0.4 µl/strip; [Myoglobin conjugate]: 0.4 µl/strip;
[CRP conjugate]: 0.3 µl/strip.

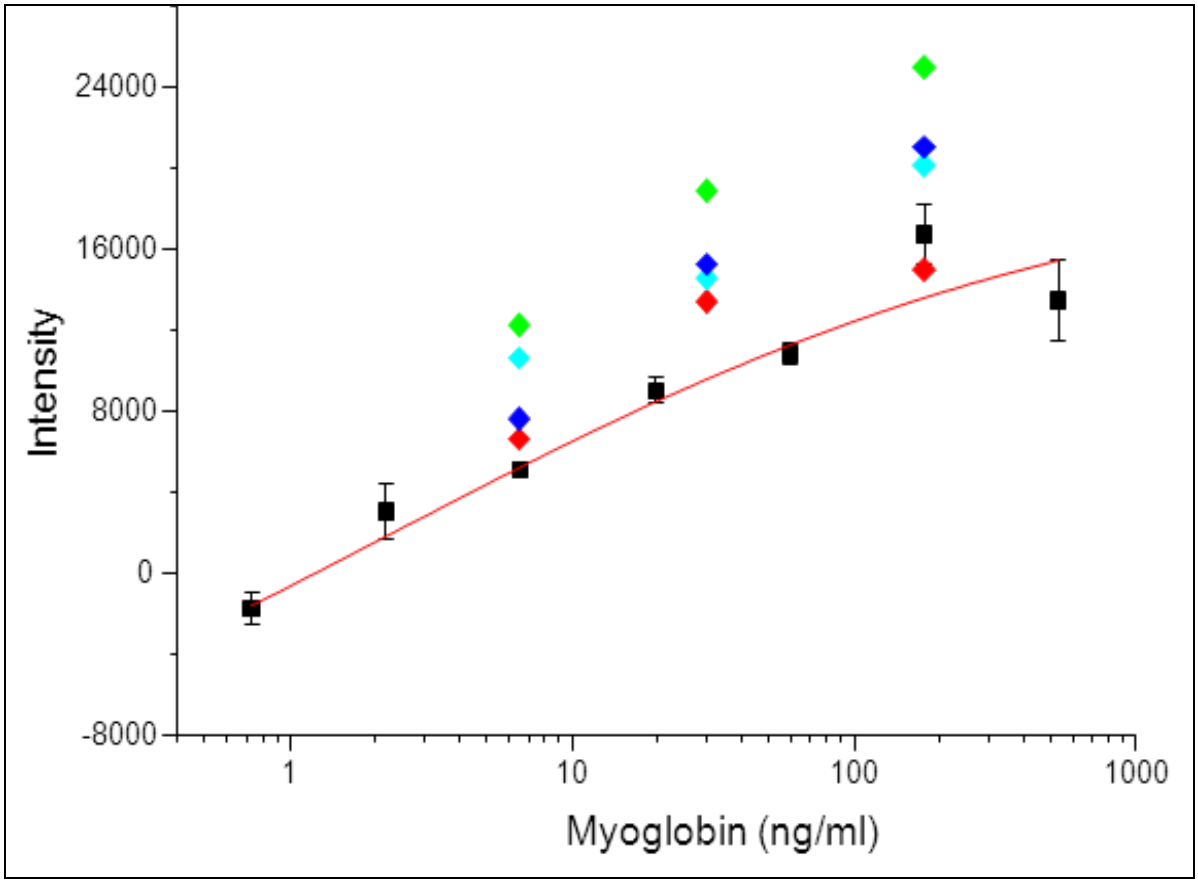


Figure 4.8: Effect of troponin and CRP on myoglobin assay at high, medium and low concentration. Myoglobin intensity read at 1335 cm^{-1} using Y-axis scan across the test line. Assay run in serum.
[CRP] ng/ml: 1800 (♦); 1800 (♦); 21(♦); 21(♦) and 0 (■).
[Troponin] ng/ml: 50 (♦); 0.54 (♦); 50 (♦); 0.54 (♦) and 0 (■).
[Troponin conjugate]: 0.4 µl/strip; [Myoglobin conjugate]: 0.4 µl/strip;
CRP conjugate]: 0.3 µl/strip.

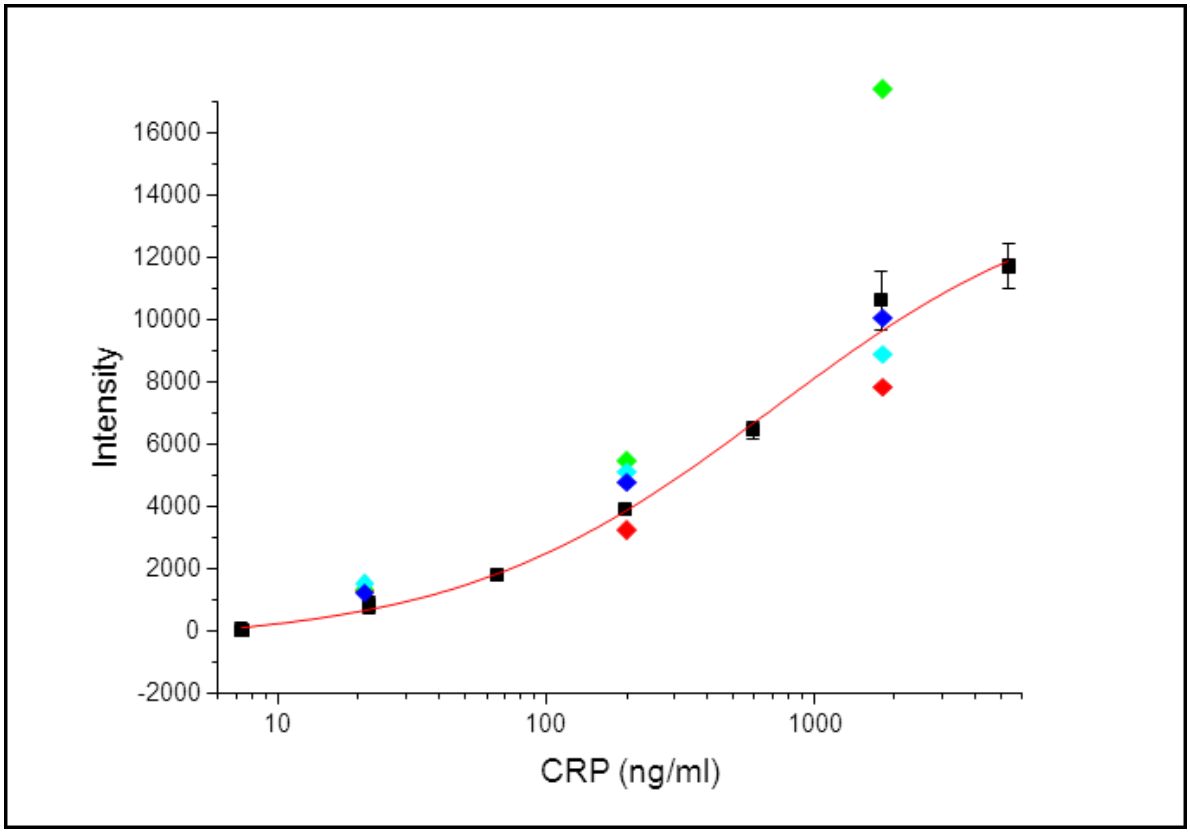


Figure 4.9: Effect of troponin and myoglobin on myoglobin assay using revised conditions. CRP intensity read at 921 cm^{-1} using Y-axis scan across the test line. Assay run in serum.
[Troponin] ng/ml: 50 (♦); 50 (♦); 0.54 (♦); 0.54 (♦) and 0 (■).
[Myoglobin] ng/ml: 178 (♦); 6.5 (♦); 178 (♦) ; 6.5 (♦) and 0 (■).
[Troponin conjugate]: 0.4 µl/strip; [Myoglobin conjugate]:0.4 µl/strip;
[CRP conjugate]: 0.3 µl/strip.

High, low analyte effect assay clearly shows that there are interferences in the assay and it need to look further in detail by analysing each candidate separately. Also troponin is most effected candidates compared to the others. It also illuminates that these results are reproducible by comparing with previous graphs (4.4 to 4.6) but it requires more improvements.

4.4 Investigation of interference in multiplexing assay

Effect of high, medium and low concentration assay (section 4.3) showed that in mixture form present of other two analyte change the intensity of target analyte. So, further assays

were undertaken to investigate the effects of the three analytes on each other's assays performance.

4.4.1 Myoglobin and CRP interference on troponin assay

Figures 4.10 to 4.12 shows typical troponin assay curves using multiplexed strips in the absences of other two analytes. Similar to previous assay troponin high, medium and low concentrations were selected to observe the effects of including CRP in the sample mixture (Figure 4.10). The addition of CRP high (3300 ng/ml) effect was grater on high concentration of troponin. It reduced the troponin signal (■) by 70%. While CRP low (51 ng/ml) reduced troponin signal (■) by ~10% of the total intensity.

Similarly, Figure 4.11 shows that addition of myoglobin high (540 ng/ml) influence was greater on the troponin intensity compare to the addition of low concentration of myoglobin (8.4 ng/ml).

A Graph 4.12 gives details the effect of CRP and myoglobin together (in mixture form) on troponin assay. Again the outcomes show the presence of high CRP (3300 ng/ml) with combination of low myoglobin (8.4 ng/ml) or high myoglobin (540 ng/ml) gives the 70% signal reduction in troponin high concentration.

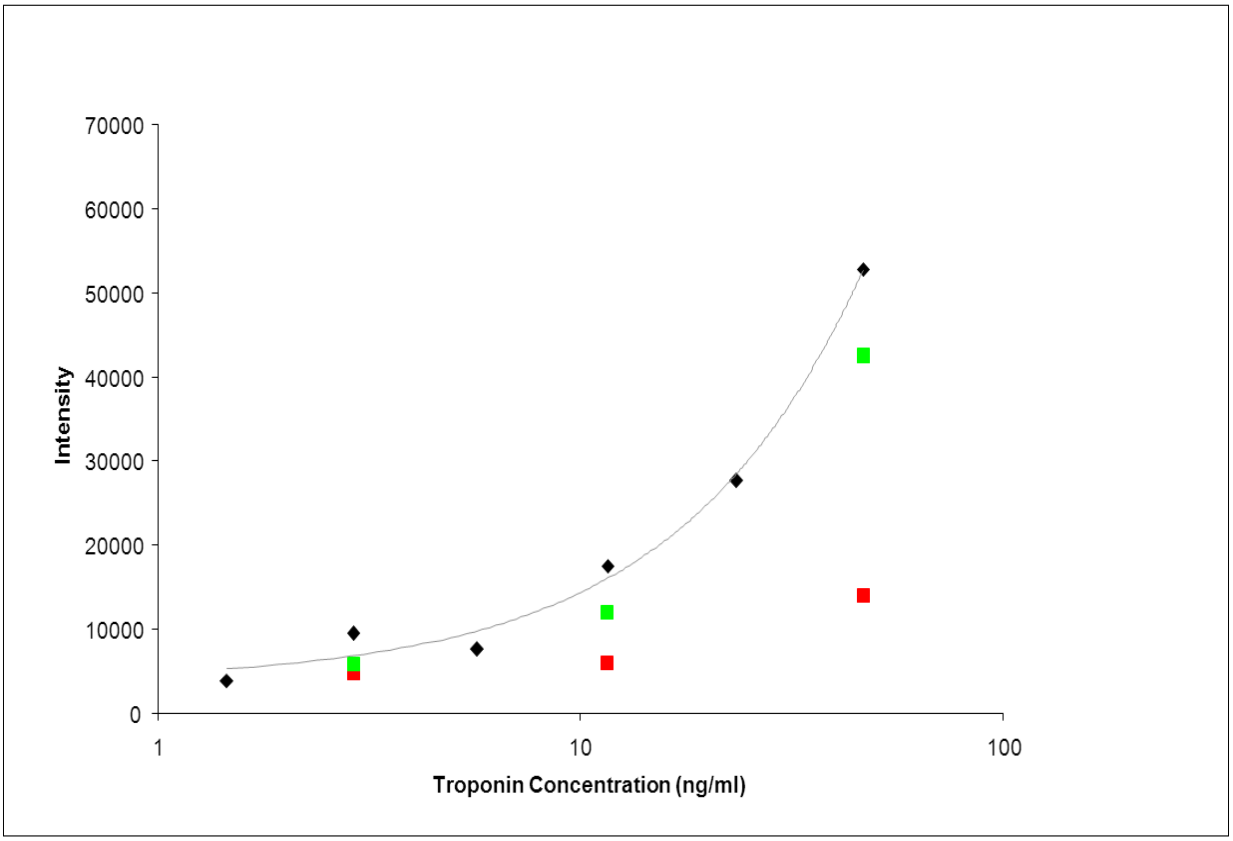


Figure 4.10: Effect of CRP on troponin assay in serum with multiplex format including both myoglobin and CRP conjugates.
[CRP] ng/ml: (♦) 0; (■) 51 and (■) 3300.
[Myoglobin] ng/ml: 0.

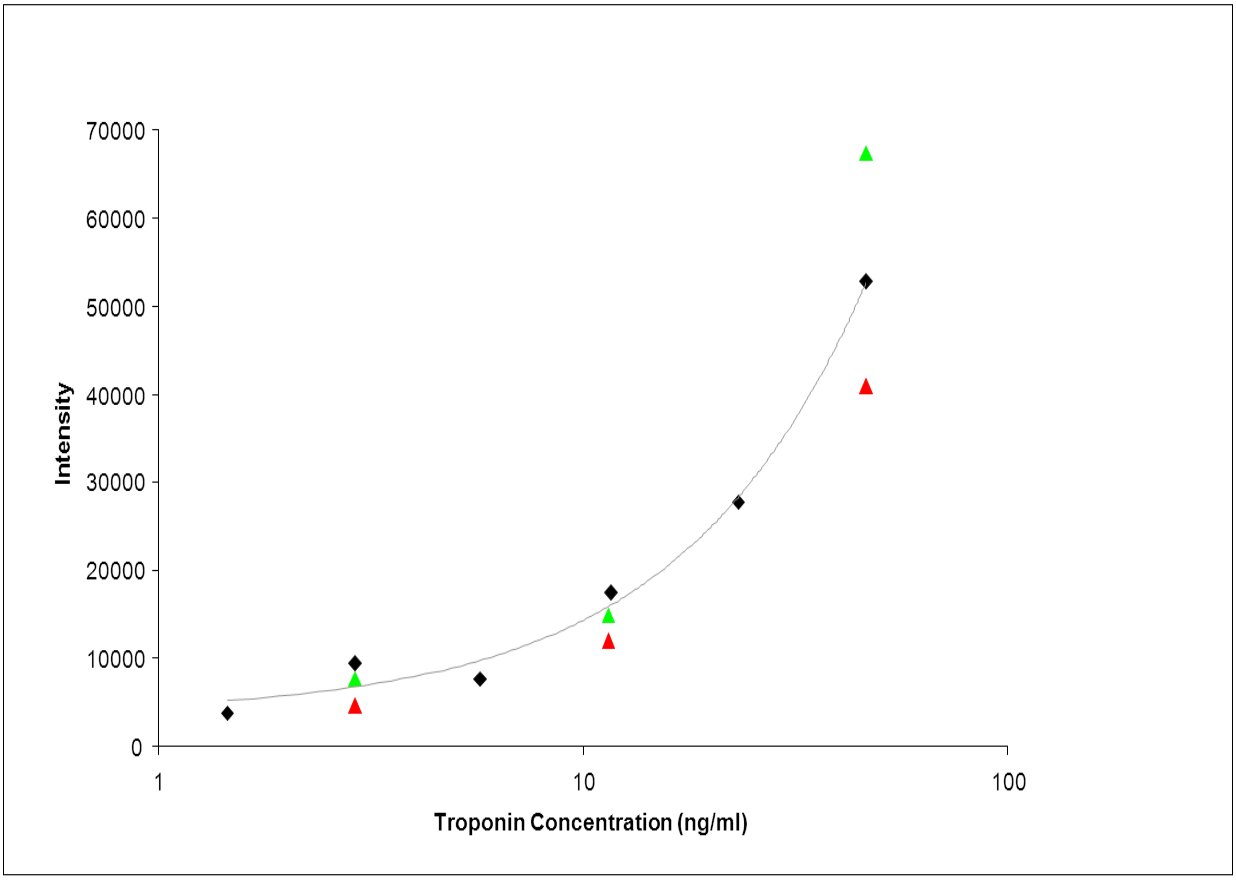


Figure 4.11: Effect of Myoglobin on troponin assay in serum with multiplex format including both myoglobin and CRP conjugates.
[Myoglobin] ng/ml: (♦) 0; (▲) 8.4 and (▲) 540.
[CRP] ng/ml: 0.

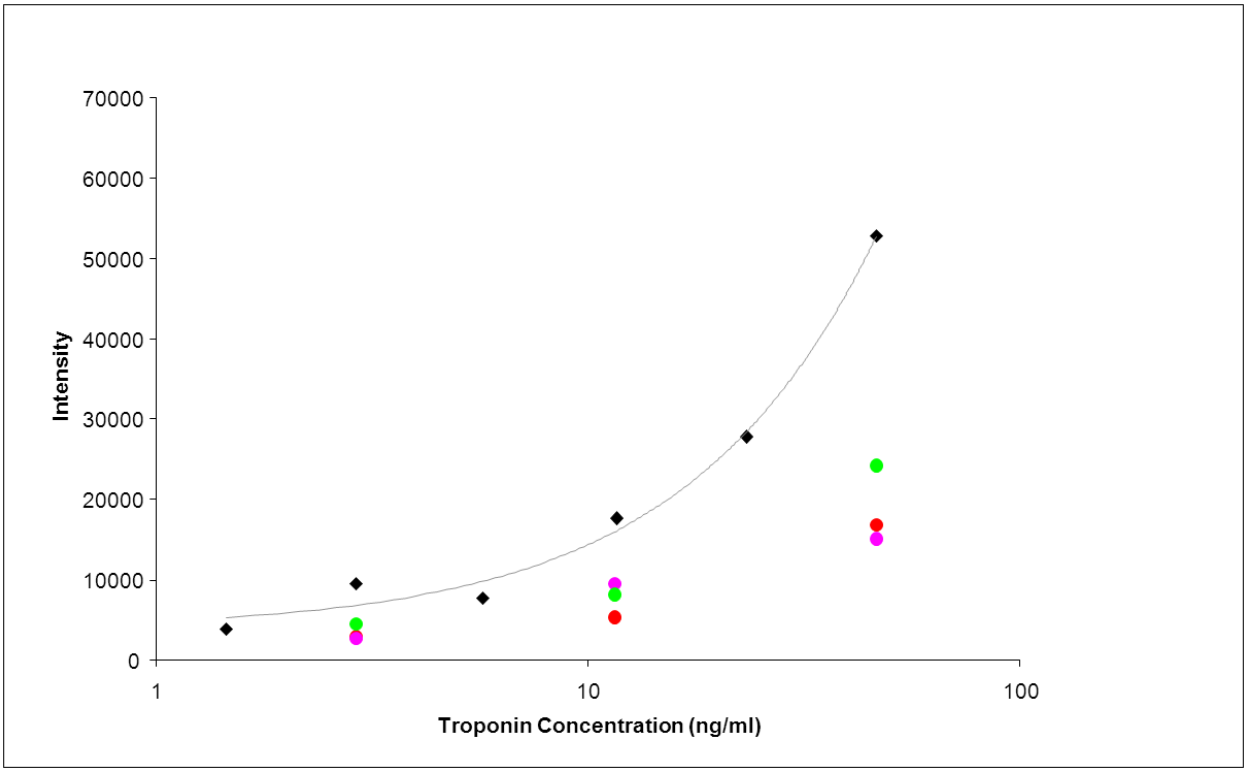


Figure 4.12: Effect of Myoglobin and CRP on troponin assay in serum with multiplex format including both myoglobin and CRP conjugates.
[CRP] ng/ml: (♦) 0; (●) 51and; (●) 3300 and (●) 3300.
[Myoglobin] ng/ml: (♦) 0; (●) 540 and; (●) 8.4 and (●) 540.

4.4.2 Troponin and CRP interference on myoglobin assay

Here Figure 4.13 to 4.15 are showing myoglobin typical curve in black line. Likely troponin curve this curve doesn't have other 2 analyte are absence but all conjugate and antibodies are present. By adding CRP low (51.5 ng/ml) on troponin assay at high, medium and low concentration it shows the reduction in intensity of myoglobin by ≈25%. On addition of high CRP (3300 ng/ml), myoglobin intensity is diminish by 50 % (▲) from the origin (■).Troponin high and low concentration effect is lower compare to CRP high effect on myoglobin assay (Figure 4.14).

When CRP and troponin together added in high concentration (●) to the myoglobin assay, myoglobin intensity is dropped by 50%. Figure 4.15 shows that when troponin concentration is low (2.9 ng/ml) and CRP kept high (3300 ng/ml) myoglobin intensity is still

not recovered (▲). On other side When troponin was kept high and CRP was added in low concentration, the effect was less but not completely removable (▼). In summary, CRP is the most influencing analyte especially when it is added in high (3300 ng/ml)

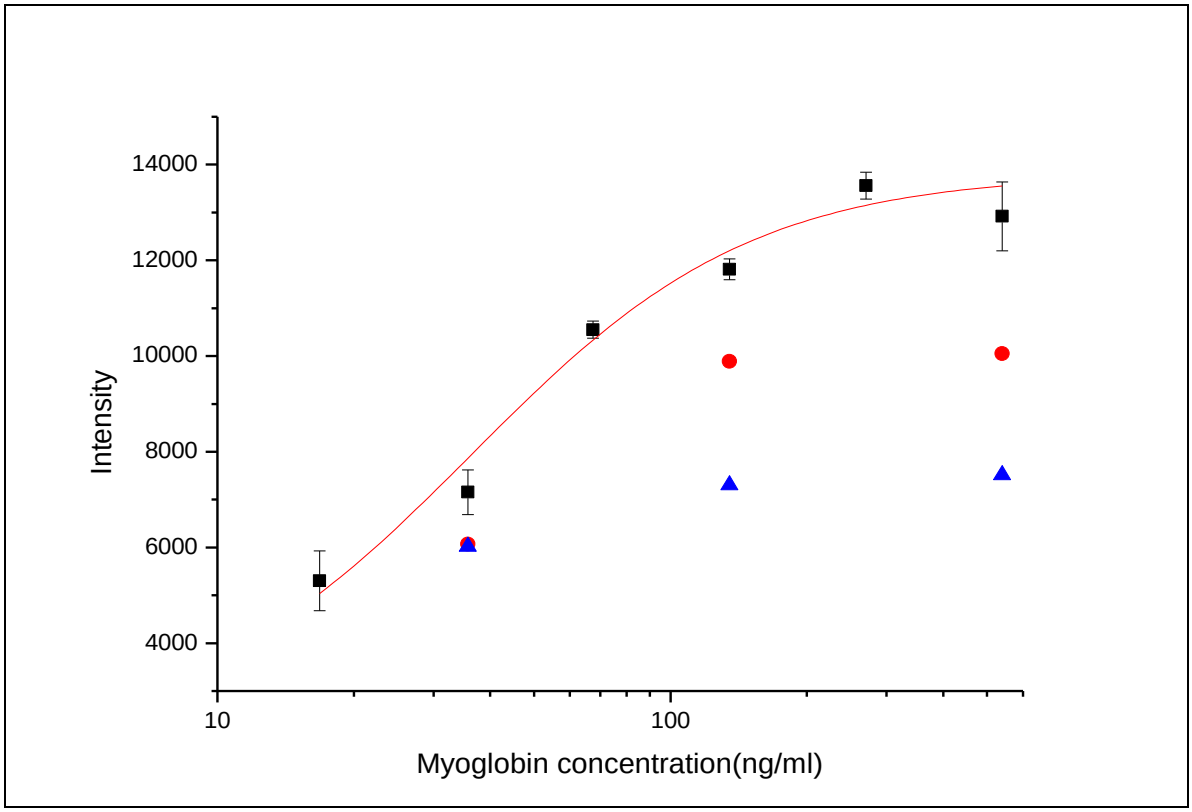


Figure 4.13: Effect of CRP on myoglobin assay in serum with multiplex format including both troponin and CRP conjugates.
[CRP] ng/ml: (▲) 3300; (●) 51 and (■) 0.
[Troponin] ng/ml: 0.

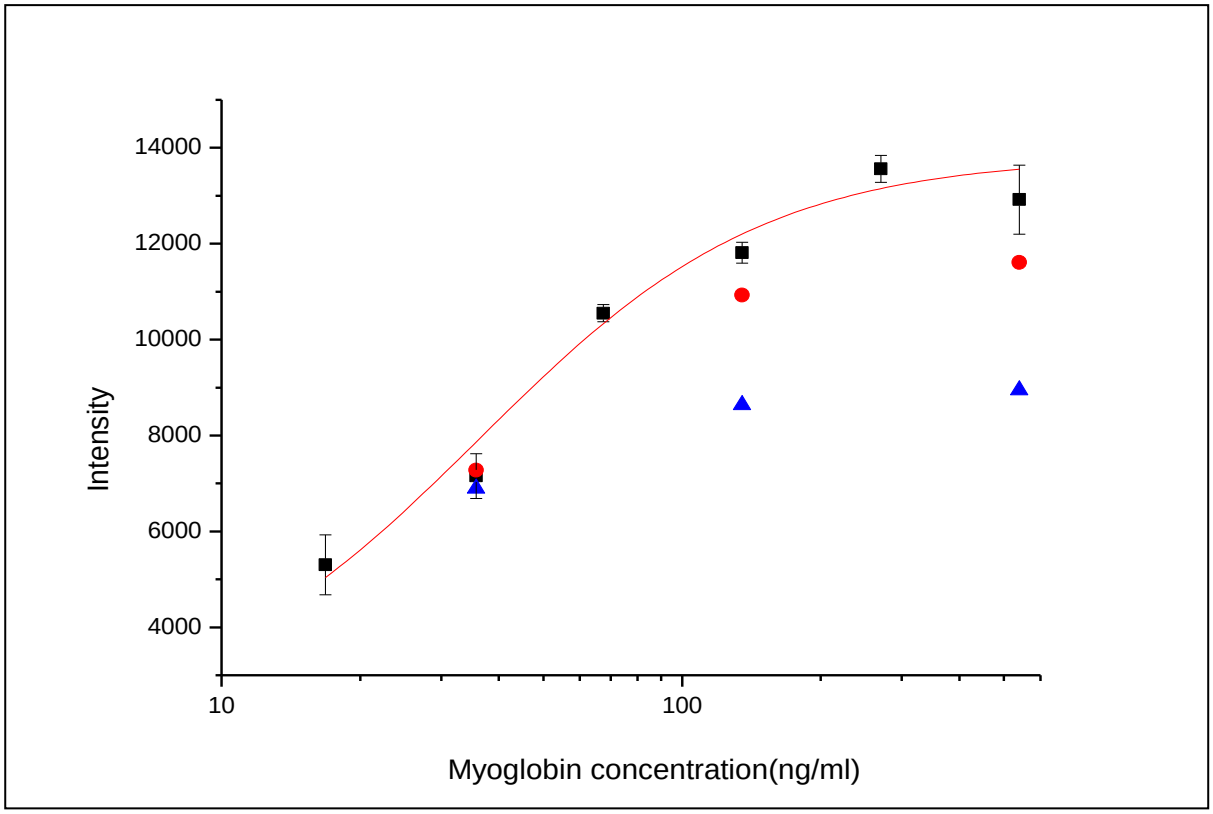


Figure 4.14: Effect of troponin on myoglobin assay in serum with multiplex format including both troponin and CRP conjugates.
[Troponin] ng/ml: (▲) 46.6; (●) 2.9 and (■) 0.
[CRP] ng/ml: 0.

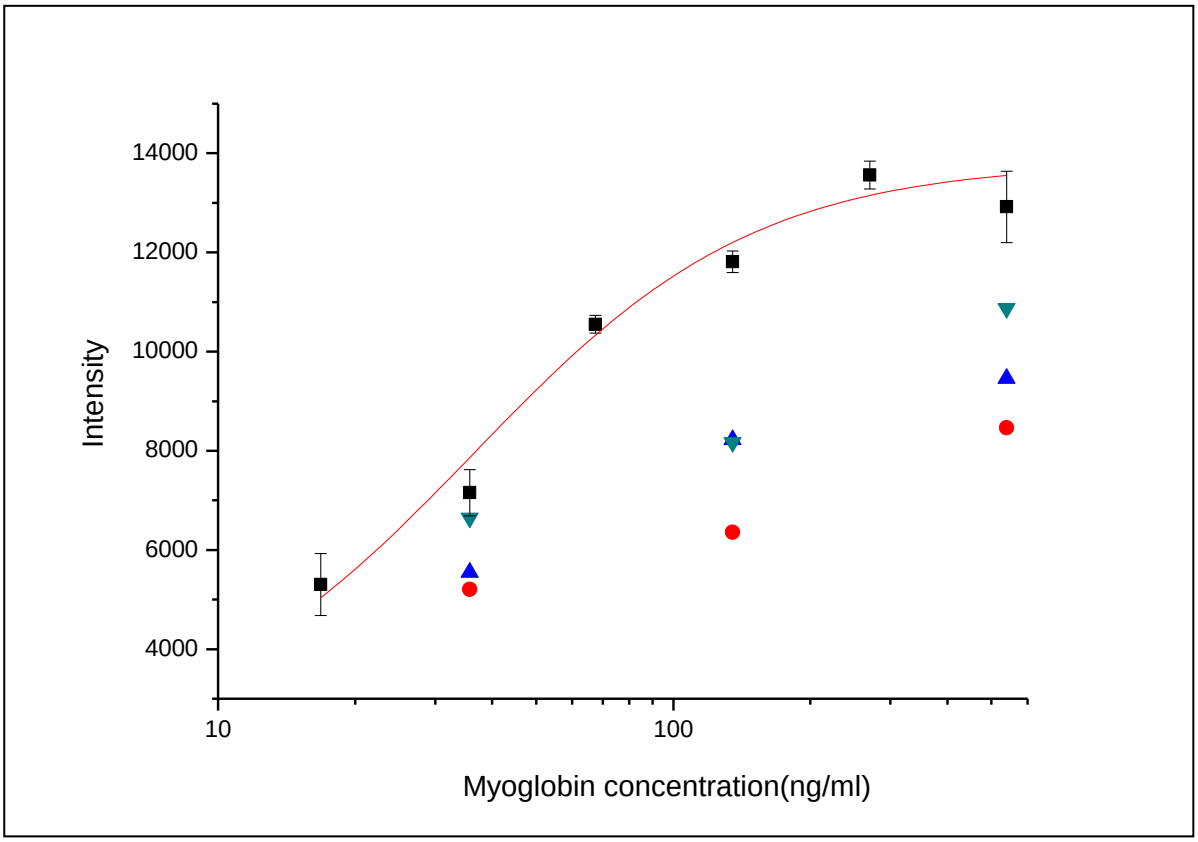


Figure 4.15: Effect of troponin and CRP on myoglobin assay in serum with multiplex format including both troponin and CRP conjugates.
[Troponin] ng/ml: (■) 0; (▲) 2.9; (●) 46.6 and (▼) 46.6.
[CRP] ng/ml: (▲) 3300; (●) 3300 ; (▼) 51 and (■) 0.

4.4.3 Troponin and myoglobin interference on CRP assay

Similar to other analytes here CRP curve is plotted in Figure 4.16 to 4.18. First myoglobin and troponin effect is observed separately in Figure 4.16 and 4.17. CRP intensity is also influenced (Figure 4.16) by adding myoglobin high (540 ng/ml). Addition of low myoglobin doesn't affect much on CRP intensity. Figure 4.17 shows troponin high and low concentration don't change CRP intensity but when it is mixed with myoglobin high and added in assay it again decrease the CRP high concentration intensity from 14000RU to 6000 RU. In overall myoglobin and troponin reduce the CRP signal as other analytes.

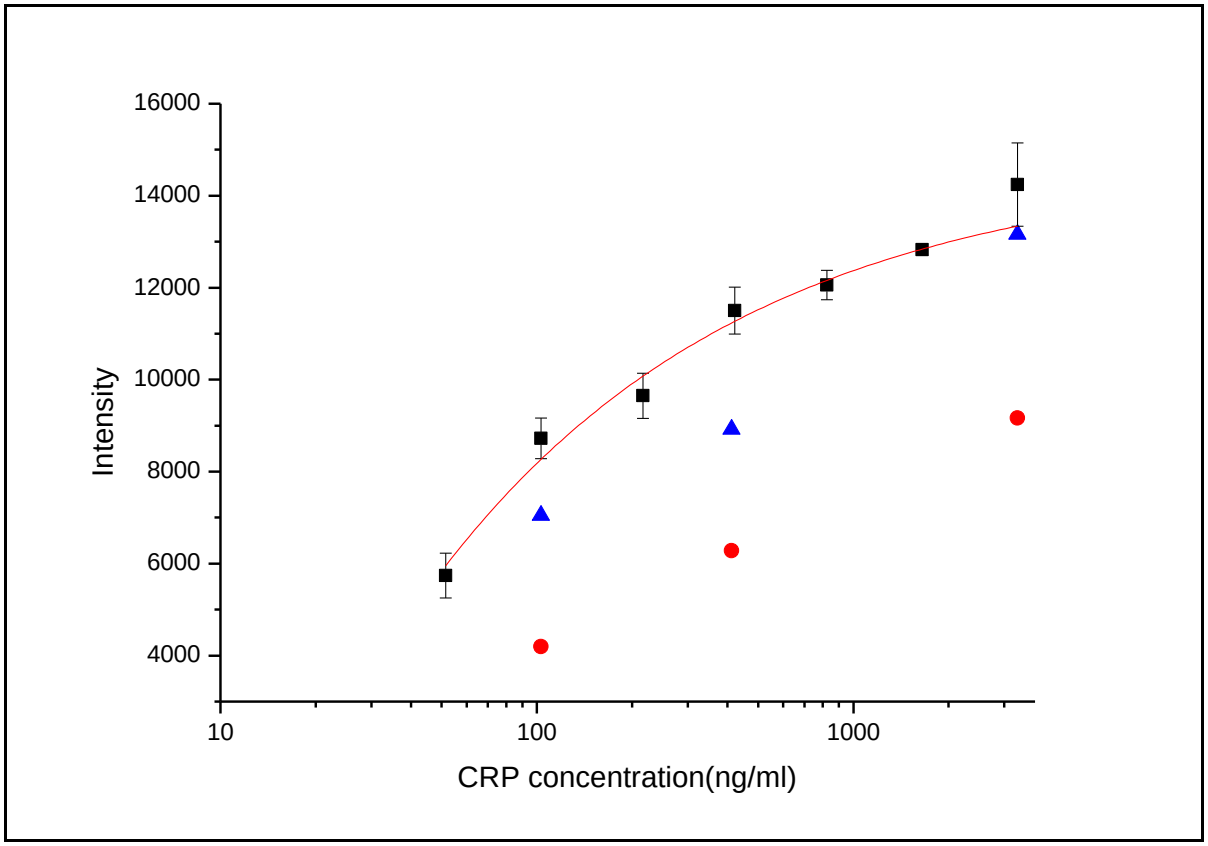


Figure 4.16: Effect of myoglobin on CRP assay in serum with multiplex format including both troponin and myoglobin conjugates.
[Myoglobin] ng/ml: (●) 540; (▲) 8.4 and (■) 0.
[Troponin] ng/ml: 0.

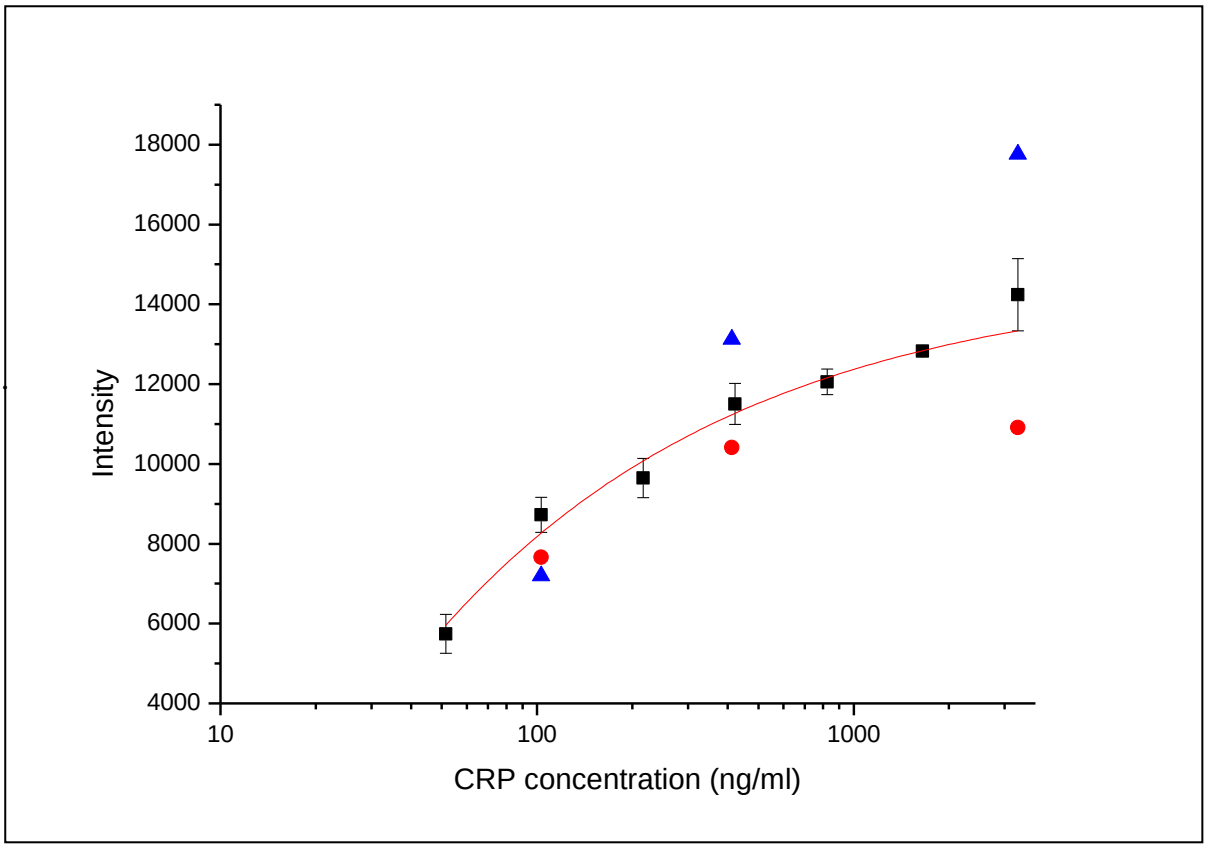


Figure 4.17: Effect of troponin on CRP assays in serum with multiplex format including both troponin and myoglobin conjugates.
[Troponin] ng/ml: (▲) 2.9; (●) 46.6 and (■) 0.
[Myoglobin] ng/ml: 0.

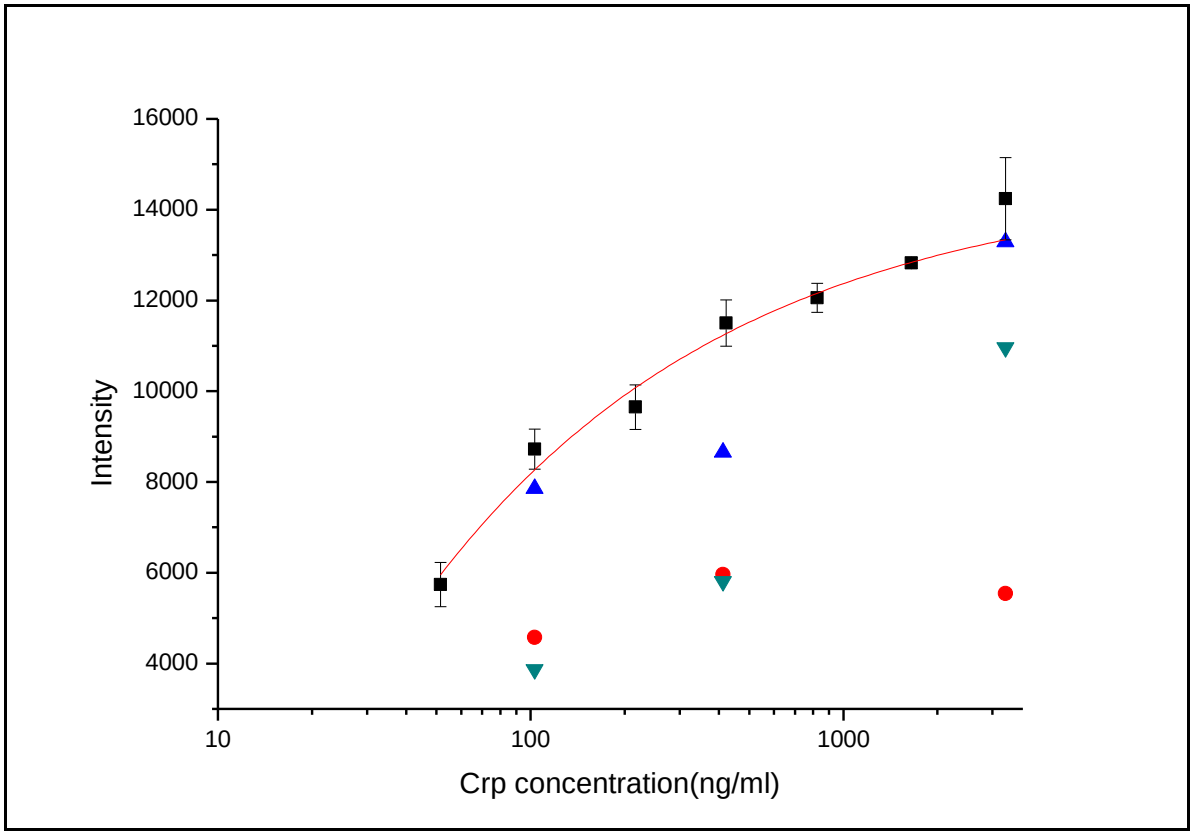


Figure 4.18: Effect of troponin and myoglobin on CRP assay in serum with multiplex format including both troponin and myoglobin conjugates.
[Troponin] ng/ml: (▲) 2.9; (▼) 2.9; (●) 46.6 and (■) 0.
[Myoglobin] ng/ml: (▲) 8.4; (▼) 540; (●) 540 and (■) 0.

4.5 Discussion

To achieve the goal of a successful multiplex assay, the examinations conducted and their outcomes are summarised here.

(1) Initial multiplexing assay on lateral flow strip in serum:

Initial multiplexed measurements were conducted using similar conditions and antibody loadings as in a simplex format. Output Raman signals were deconvoluted to read separate Toponin intensity at 1159 cm^{-1}, myoglobin at 1335 cm^{-1} and CRP at 921 cm^{-1}. Unfortunately, the outcomes indicated that the Raman intensity of troponin was very low as compared to the myoglobin and CRP intensities. In addition, compared to the single analyte assay (section 3.8) the 'quality of the Raman signal' of troponin and myoglobin was poorer, *i.e.*

very low intensity was observed for all concentrations in the multiplex assay (section 4.1). This output indicated that the three selected analytes are not successfully detected in mixed form as they are detected individually. Consequently, the refinement of assay conditions was targeted in this chapter.

(2) Refinement of multiplexing assay in serum:

Improvement of the multiplexed assay was achieved in section 4.2. In order to gain an improved Raman intensity especially for troponin and myoglobin, the assay was performed by increasing their conjugate loading of troponin:myoglobin:CRP ratio from 1:1:1 to 1.5:1.5:1. This modification of the assay made the chosen biomarkers separately detectable using multiplex format on a single test line of the lateral flow strip. Each biomarker detection range in the presence of co-analytes in serum was as below: CRP was measurable from 5 ng/ml to 6000 ng/ml; troponin was measurable from 0.1 ng/ml to 50 ng/ml; and myoglobin was measurable from 0.5 ng/ml to 600 ng/ml.

(3) Effect of high, medium and low concentration of analytes in multiplexing assay:

Section 4.3 describes the measurements of the effect of high, medium and low concentration of co-analyte in the multiplex assay. The results were overlaid on the outcomes achieved in section 4.2 to identify the interference effect. However, the results of each analyte assay were disappointing when the concentrations of the other two analytes were altered in the assay. Targeted analyte intensity was inhibited by the addition of high or low concentration of the co-analytes in the assay. Troponin was the most significantly affected biomarker of the three. It was also seen that the multiplexing results were reproducible by comparing with previously done calibrations in section 4.2. The inhibition effect of co-analytes did not give any specific information about its origin. Nonetheless, it highlighted that the interference issues could arise for real disease samples. Hence,

individual analyte measurements require thoughtful assessments. To address this issue a further investigation was designed.

(4) Investigation of interference in multiplexing assay:

Investigation of the interference issue was carried out for individual analytes in section 4.4. Individual analyte curve was obtained on a multiplex strip (all three capture antibodies were present) by loading all three conjugates with only targeted analyte. Then, to identify the interference single co-analytes were added in the assay in high, medium and low concentration of target analyte. The results showed that CRP interferences on troponin Raman intensity were more significant than myoglobin interference. The combined addition of CRP and myoglobin showed almost 50% to 60 % signal drop in troponin intensity due to the presence of CRP (section 4.4.1). Similarly, a higher CRP interference was detected on myoglobin intensity as compared to the troponin interference. Troponin and CRP impact in mixture was visible on myoglobin by the drop of Raman signal by 50 % (section 4.4.2). At last, the investigation of CRP intensity showed that the myoglobin interference was twice compared to the troponin interference. Troponin and myoglobin mixture effect showed almost 60 % decrease in CRP Raman intensity (section 4.4.3).

In conclusion, this chapter has shown the potential of SERS particles by establishing multiple measurements in single test line in serum. However, the quantitative analysis showed the challenges of co-analytes' interferences. Deeper investigations demonstrated that the CRP was the most influential candidate for causing interference while troponin was the most affected among the three biomarkers. More investigations are needed understand the cause of these interferences.

CHAPTER 5: Investigation of interference issues

5.0 Introduction

The proficiency of the SERS particles to identify individual biomarker from a complex mixture was demonstrated in chapter 4 by establishing a multiplex assay. Nevertheless, further quantitative examination revealed the challenge of considerable interference of co-analytes in measurements of the targeted analyte's Raman signal. The source of the interference needs to be identified to eliminate it from the multiplex assay. Therefore, the aim of this chapter was to investigate the fundamental cause of the interference problem and thus achieve a quantitative assay. Part of this work was undertaken and published with colleagues in the Biotechnology department of the National Physical Laboratory. Literature review has suggested that interferences in the immunoassay can arise from the various sources, like nanoparticles or immunological, depending of the assay design ((b) Davies, 2005; Kelly *et al*., 2003). After carefully considering these a number of possible mechanisms were identified to inspect the cause of the interference issue:

(1) Non-specific interactions between co-analytes and antibodies:

Antigen – antibody reaction plays a central role in lateral flow immunoassay. Thus, to develop a robust lateral flow test, the required antibody should be highly specific and should have a high affinity for the target antigen. Aggregation of particles, denatured or polyclonal antibody or the impurity of the material (antibody or antigen) can result in cross-reactivity that can be seen as either loss of signal or incorrect signal because of the binding of conjugates to the test line in the absence of their specific analyte ((b) Davies, 2005). Even though highly specific (monoclonal) antibody and antigen were purchased for this work but still the possibility of cross-reactivity is quite high during the process of conjugation or

sample running in mixture. Therefore, it is crucial to examine the non-specific interactions in the assay. With this goal in mind, assays were performed to identify the cross-reactivity between the target analyte and co-analytes or between their antibodies. Section 5.1 describes all examined and non-examined but considered mechanisms of the cross-reactivity.

(2) "Steric hindrance/crowding" between the SERS nanoparticles on the test line:

Steric hindrance occurs when a large group of particles inhibit a chemical reaction in a small available space by competing with each other. Very few studies have investigated the concept of steric hindrances between the particles. Previous examination (section 4.4) had showed that when CRP-421 SERS particles were added in high concentration to plot the troponin calibration curve, it led the inhibition of troponin Raman intensity. Hence, the purpose of this examination was to identify the displacement of the troponin conjugate (M19C7- 470 SERS) or blocking of the troponin capture antibody binding site due to the presence of CRP in high concentration. To understand this artefact a two-run assay was performed using troponin as a model biomarker. Troponin and CRP/myoglobin assay were run one after the other. A scanning electron microscope (SEM) characterisation was carried out to investigate the crowding effect or the space on the test line. Therefore, a multiplex assay test line with all three analytes at maximum concentrations (troponin 50 µg/L, myoglobin 500 µg/L and CRP 50 mg/L) was imaged by SEM to quantify the number of particles on the strip. Furthermore, a test-strip with about three time higher concentration of anti- mouse antibody - 470 particles was examined by SEM to compare with the multiplex strip. The outcomes are presented in section 5.2.

(3) Optical Interference:

Recent articles have investigated the optical properties of the metal nanoparticles including calculation of scattering, absorption and excitation efficiencies and their role in biological detection (Doering *et al*., 2007; Kelly *et al*., 2003; Stuart *et al*., 2005). Knowledge of the extinction coefficient and scattering behaviour of a SERS particle upon the formation of a biological over-layer is important for a better understanding of the operation of biosensors. Therefore, the purpose of this section was to examine if the high scattering property of the gold nanoparticle limits light penetration into the strip and causes the interference.

To understand the mechanism of the interference between incident laser photons and Raman scattered light, the assays were made simple by analysing in duplex form without the addition of any antibody or analyte. Therefore, a mixture of 421 and 470 SERS particles were deposited on test line to identify the loss of Raman intensity, corresponding to the increase in concentration of the second particle (Figure 5.9). To further understand this effect, Raman intensity of single (470) SERS particles was monitored after successive addition of similarly sized naked gold nanoparticles in the assay. Optical properties of the SERS and gold nanoparticles was calculated from Mie theory, the density was measured from the SEM images and the concentration was estimated from absorbance at 520 nm to identify the optical interference in the output Raman intensity. The observed optical interferences and the way to eliminate it from multiplex assay are explained in section 5.3.

5.1 Non-specific interactions between co-analytes and antibodies

In multiplexed assays there is the potential for non-specific interactions between co-analytes and either or both of the capture and detection antibodies of other analytes. For example,

$$Ab_2\text{- SERS(sol)} + Ab_1\text{(im)} \rightleftharpoons (Ab_2\text{-SERS})\, Ab_1\text{(im)} \qquad \text{(A)}$$

and/or

$$Ag_2\, Ab_2\text{-SERS(sol)} + Ab_1\text{(im)} \rightleftharpoons (Ag_2Ab_2\text{–SERS})Ab_1\text{(im)} \qquad \text{(B)}$$

Equation: (A) shows the non-specific interaction between the capture antibody (Ab_1) for the primary analyte and the detection antibody–SERS particle conjugate in for a co-analyte in the absence of the co-analyte. Similarly, (B) shows the non-specific interaction between the capture antibody (Ab_1) for the primary analyte and the detection antibody- SERS conjugate (Ab_2-SERS) with co-analyte (Ag_2). Where the bracketed terms aq, im and sol indicate aqueous, immobilised and sol phases, respectively.

To demonstrate whether or not either of both of these mechanisms were significant in this work, assays were carried out using nitrocellulose strips with single analyte capture antibodies and run with the two co-analytes (test) and principal analytes (control) using the normal test regime (section 2.5). Results are shown in Figure 5.1 and the assay conditions are summarised in Table 5.1.

Tests 1 to 6 in Table 5.1 are equivalent to the simplex assays described in chapter 3 (section 3.7) and gave the expected results, *i.e.* in the presence of the target analyte, both the control and test lines are positive; while in the absence of the target analyte the test line is negative (no signal) and the control line is positive. Tests 7, 9 and 11 show no (or negligible) interaction between co-analyte/antibody conjugates and the analyte capture antibodies (equation (B) above). Tests 8, 10 and 12 show no interaction between capture antibodies from the test line to co-analyte detection antibody as above in equation (A).

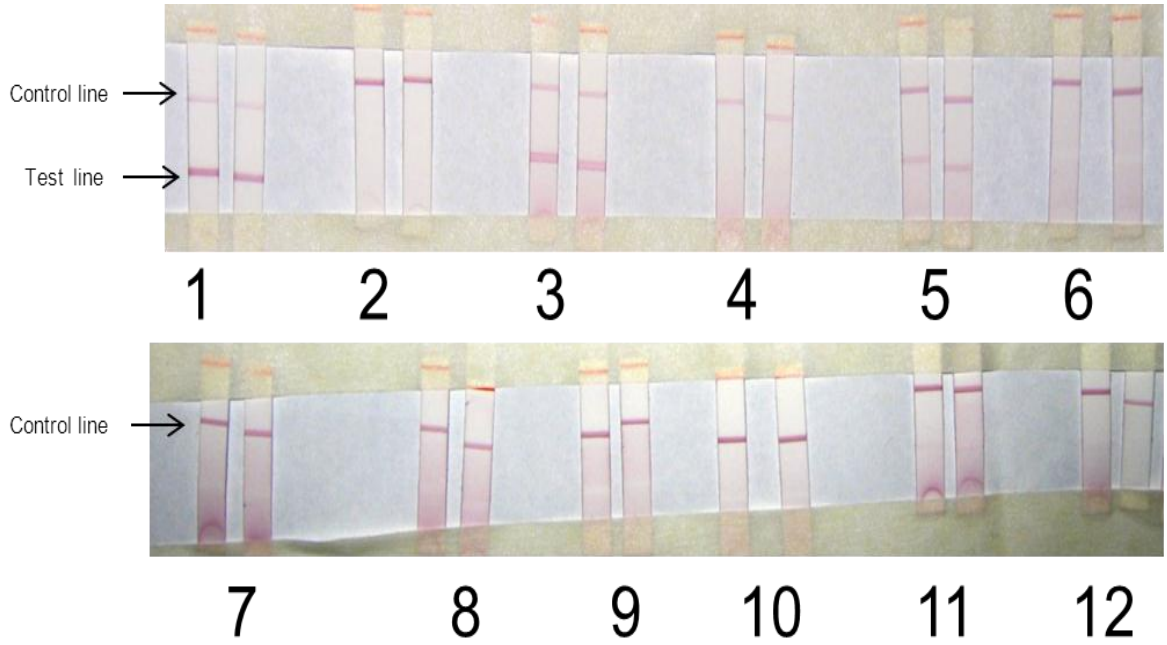


Figure 5.1: Assays to look for non-specific interactions between co-analytes and antibodies. Capture antibody (Ab_1): (1, 2, 7, 8) CRP; (3, 4, 9, 10) myoglobin; and (5, 6, 11, 12) troponin. Antibody conjugate(s) (Ab_1-SERS): (1, 2) CRP; (3, 4) myoglobin; (5, 6) troponin; (7, 8) myoglobin and troponin; (9, 10) CRP and troponin; (11, 12) CRP and myoglobin.
Test protein (Ag_i): (1) CRP; (2, 4, 6, 8, 10, 12) none; (3) myoglobin; (5) troponin; (7) myoglobin and troponin; (9) CRP and troponin; and (11) CRP and myoglobin.

There are two other non-specific interaction modes that were not investigated [equations (C) and (D)] but, there is no reason to suppose that they are likely to be more significant than the interactions that were tested.

$$Ag_2(aq) + Ab_1 - SERS(sol) \rightleftharpoons Ag_2Ab_1\text{-}SERS(sol) \qquad (C)$$

and/or

$$Ag_2(aq) + Ab_1(im) \rightleftharpoons Ag_2Ab_1(im) \qquad (D)$$

All possibilities of non-specific cross reactions (equations A to D) are demonstrated in Table 5.1 for troponin, myoglobin and CRP.

No[a]	Capture antibody	Analyte(s)		Conjugate		Testline	Mechanism
	Ab_1(im)	Ag_1(aq)	Ag_2(aq)	Ab_1-SERS(sol)	Ab_2-SERS(sol)		tested
1	CRP	CRP	-	CRP	-	+'ve	-
2	CRP	none		CRP	-	-'ve	-
3	myoglobin	myoglobin	-	myoglobin	-	+'ve	-
4	myoglobin	none		myoglobin	-	-'ve	-
5	troponin	troponin	-	troponin	-	+'ve	-
6	troponin	none		troponin	-	-'ve	-
7	CRP	-	myoglobin +troponin	-	myoglobin +troponin	-'ve	B
8	CRP	none		-	myoglobin +troponin	-'ve	A
9	myoglobin	-	CRP +troponin	-	CRP +troponin	-'ve	B
10	myoglobin	none		-	CRP +troponin	-'ve	A
11	troponin	-	CRP+ myoglobin	-	CRP +myoglobin	-'ve	B
12	troponin	none		-	CRP +myoglobin	-'ve	A
NT	CRP	-	myoglobin	CRP	-	*	D
NT	CRP	-	troponin	CRP	-	*	D
NT	myoglobin	-	CRP	myoglobin	-	*	D
NT	myoglobin	-	troponin	myoglobin	-	*	D
NT	troponin	-	CRP	troponin	-	*	D
NT	troponin	-	myoglobin	troponin	-	*	D
NT	CRP	CRP	myoglobin	CRP	-	**	A,B,C
NT	CRP	CRP	troponin	CRP	-	**	A,B,C
NT	myoglobin	myoglobin	CRP	myoglobin	-	**	A,B,C
NT	myoglobin	myoglobin	troponin	myoglobin	-	**	A,B,C
NT	troponin	troponin	CRP	troponin	-	**	A,B,C
NT	troponin	troponin	myoglobin	troponin	-	**	A,B,C

Table 5.1: Assay conditions to look for non-specific interactions between co-analytes and antibodies.

[a]No refers to the strip number in Fig. 5.1. NT indicates 'not tested'.

* A positive testline response would be expected if mechanism D was significant.

** A less intensely positive testline would be expected if mechanism C was significant.

(NOTE: Conceptually, mechanisms A and B could apply simultaneously in this scenario.)

5.2 "Steric hindrance/crowding" between the SERS nanoparticles on test line

SERS nanoparticles are ~90-150 nm in diameter. Therefore the possibility of binding conjugate particles at the test line is limited by the space available for these large particles. Figure 5.2 shows that if more particles (conjugates) are loaded on the test line then there is a competition between the particles and they can physically block the access for another analytes conjugates to bind on a test line.

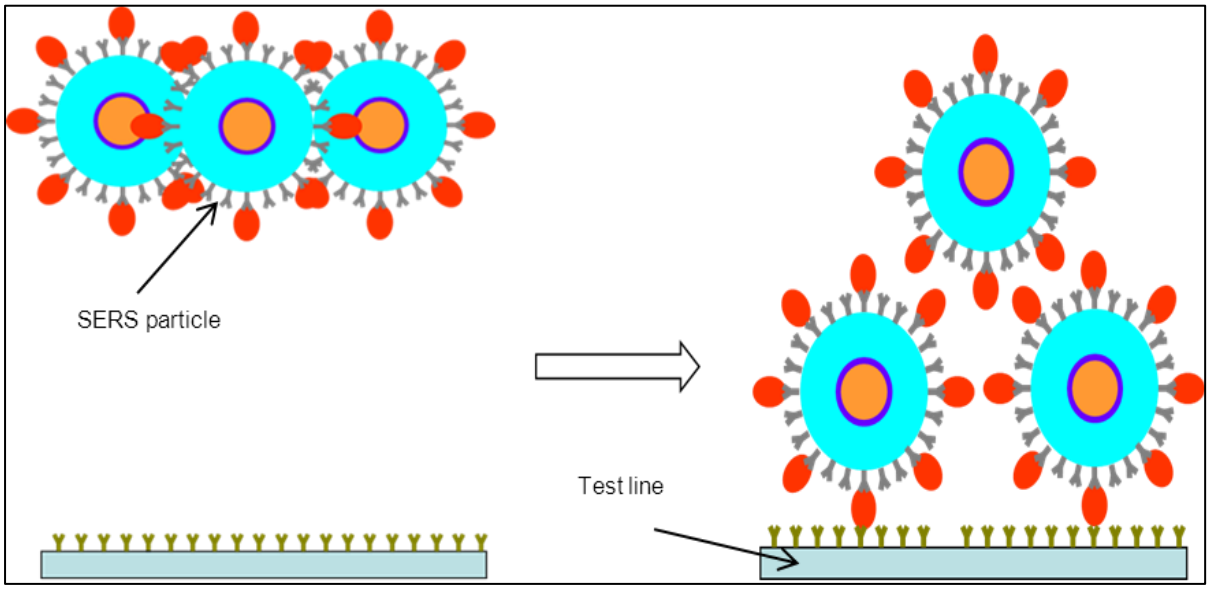
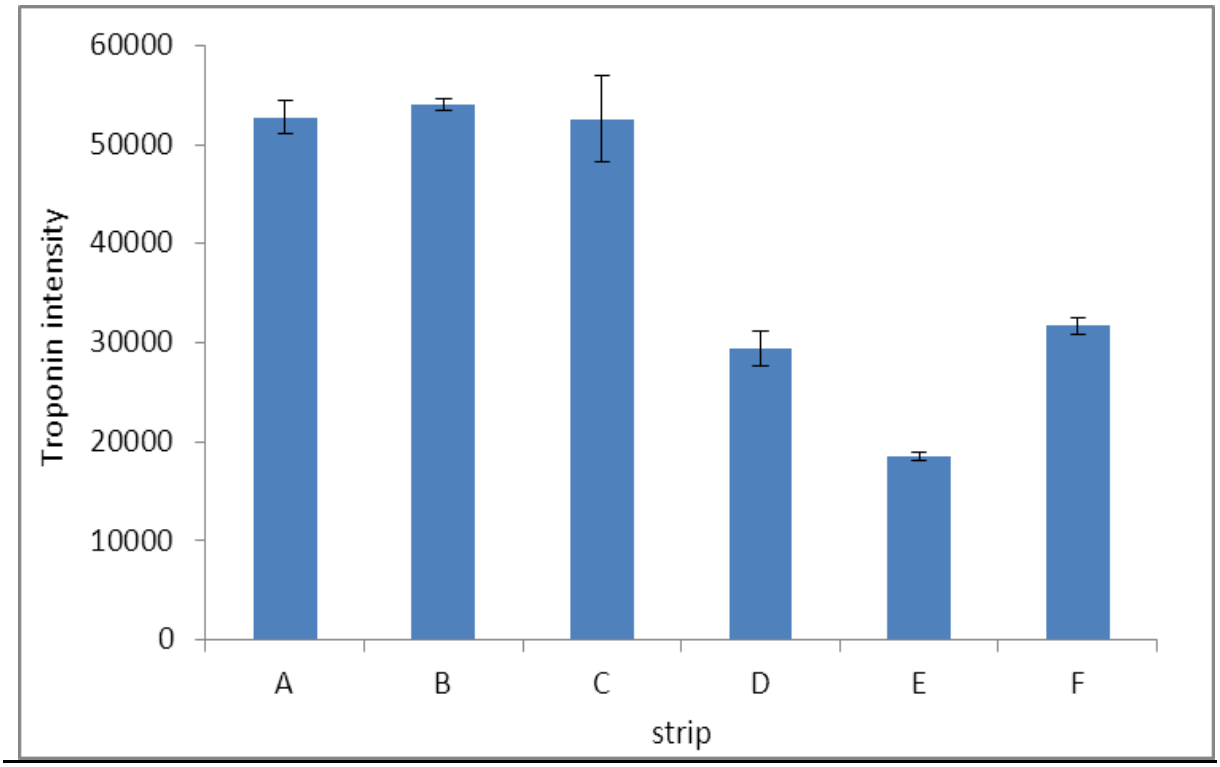


Figure 5.2: Schematic of the SERS stric effect of lateral flow strip. (Left) large amount of SERS partilces coming on test line. (Right) when they bind on a test line some particles don't have space to attach due to other partilces hindering the space.

Therefore "two run" experiments were structured by running separately each analyte as mentioned in chapter 2 (section 2.10) on the same lateral flow strip and observing the signal of the first analyte. Troponin was the most affected analyte, therefore it was chosen as a model for this assay. Figure 5.3 shows the Raman signal of troponin on the Y axis and the X axis are strips A to F. Multiplexing strips were used for this assay. Here strip A is adopted from the figure 4.10 to compare with strip B that is having serum as first rum. Strip B shows highest intensity; it has serum as a first run and troponin assay as a second run. It proves there is no effect of addition of serum in the assay as a first run. Even strip C is showing

similar intensity which had CRP analyte run without conjugate as a 1st run before the troponin run on a strip. This indicates no analyte effect on the Raman intensity of troponin and no cross reaction between the troponin and CRP antibodies. But when both conjugates were mixed and run with the CRP analyte in strip E before the troponin run, it might be blocking the binding site of troponin because it reduces the troponin signal by 40%. On strip E both assays were run together and troponin intensity was recorded similar to strip D. In strip F first run was a serum and second run was a mixture of troponin and CRP, but no changes occurred in the result. Troponin intensity from the strip F was recorded similar to strip D. List of the analytes run in the assay are also recorded in figure 5.3.

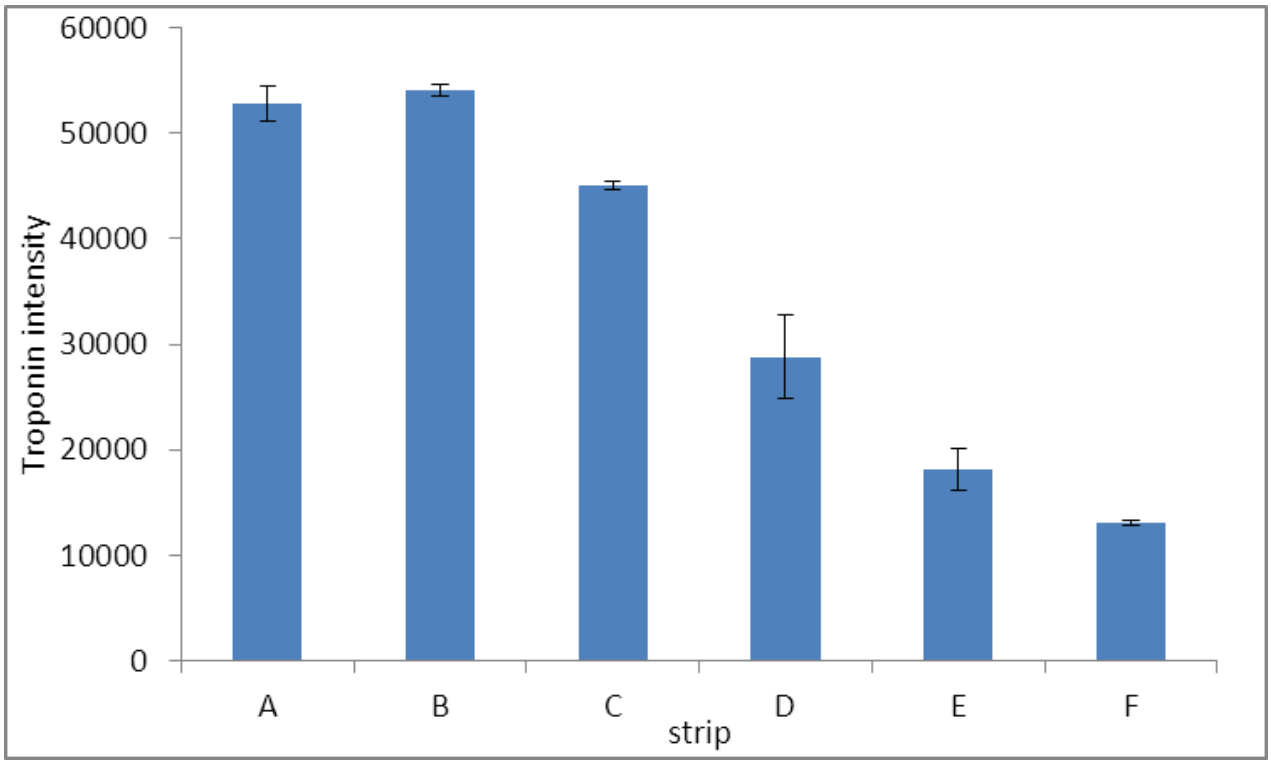


Strip	Run 1	Run 2
A	Troponin(analyte+ conjugate)	Serum
B	Serum	Troponin (analyte + conjugate)
C	CRP (analyte)	Troponin (analyte + conjugate)
D	CRP (analyte + conjugate)	Troponin (analyte + conjugate)
E	CRP (analyte + conjugate) + Troponin (analyte + conjugate)	—
F	Serum	CRP (analyte + conjugate) + Troponin (analyte + conjugate)

Figure 5.3: CRP interference on troponin intensity using multiplex strip (all captured antibodies are present). Troponin intesity was measured at 1159 cm^{-1} using Y-axis scan across the test line. Table includes list of analytes and conjugates used in strip A to F.
[CRP] ng/ml: 9900;
[Troponin] ng/ml: 150;
[Myoglobin] ng/ml: 0.

Figure 5.4 strip A is also adopted from the figure 4.11 for comparing with serum addition as a first run. Strip B shows the exact same intensity as the previous assay. It has the highest intensity. It also proves the reproducibility of the assay and no serum effect. Strip C shows slightly less intensity which may be because of myoglobin analyte run before running

troponin assay. Strip D is gives almost half signal compare to strip A and B. That may be due to myoglobin blocking the sites on the test line for troponin binding. Troponin signals on strip E and F were significantly low where myoglobin and troponin run together before and after running serum. These experiments showed the troponin signal is lower after running a CRP or myoglobin assay.



Strip	Run 1	Run 2
A	Troponin(analyte +conjugate)	Serum
B	Serum	Troponin (analyte + conjugate)
C	Myoglobin (analyte)	Troponin (analyte + conjugate)
D	Myoglobin (analyte + conjugate)	Troponin (analyte + conjugate)
E	Myoglobin (analyte + conjugate) + Troponin (analyte + conjugate)	—
F	Serum	Myoglobin (analyte + conjugate) + Troponin (analyte + conjugate)

Figure 5.4: Myoglobin interference on troponin intensity using multiplex strip (all captured antibodies are present). Troponin intesity was measured at 1159 cm^{-1} using y-axis scan across the test line.Table includes list of analytes and conjugates used in strip A to F.
[Myoglobin] ng/ml: 1620;
[Troponin] ng/ml: 150;
[CRP] ng/ml: 0.

The other evidence by SEM images showed that steric hindrance is not nor the clogging is presence on the strip. All high concentration (multiplexed) strips were tested on SEM that shows plenty of spaces on the test line for binding more particles. Also in addition to that 470 particles with anti-mouse antibody on the test-line were tested and shown three fold higher particles in the image. These images confirm that number of particles binding to the test line does not clog all pores or cover nitrocellulose surface (James et al., 2012). In summary, provided results shows that steric hindrance may not be the cause of interference. Figure 5.5 is a pre-test line where no SERS particles are present. Figure 5.6 is the test line with all three (troponin, myoglobin and CRP) analytes in highest concentration and Figure 5.7 has 470 particles with anti-mouse antibody to access the maximum no of capture binding sites. The total no of particles counted by SEM on the strip were threefold higher than the no of particle counted on the strip from Figure 5.6.

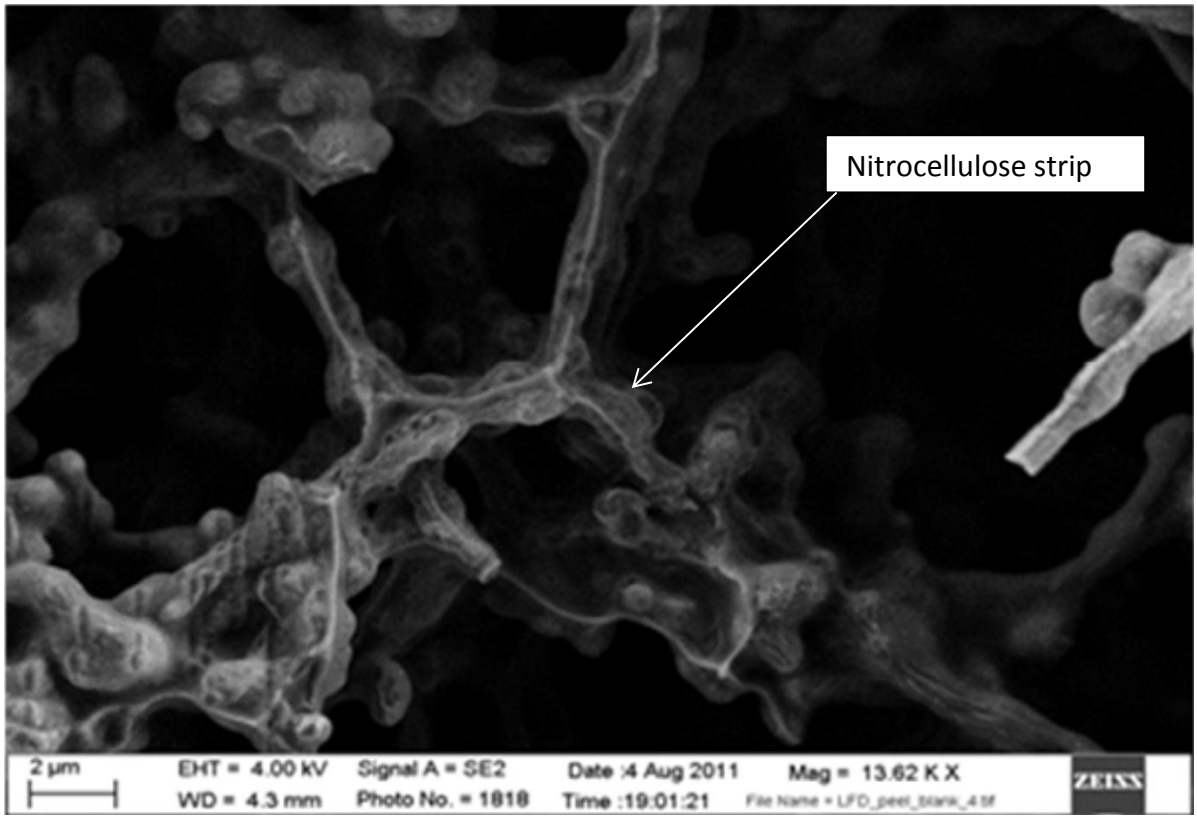


Figure 5.5: Pre-test line of nitrocellulose strip without any SERS particles. Reprinted with permission from Noble, J., Attree, S., Horgan, A., Knight, A., Kumarswami, N, Porter, R., and Worsley, G. (2012). Optical scattering artifacts observed in the development of multiplexed surface enhanced Raman spectroscopy nanotag immunoassays. *Anal. Chem.* **84**, 8246-8252 Supplementary information. Copyright 2012 American Chemical Society.

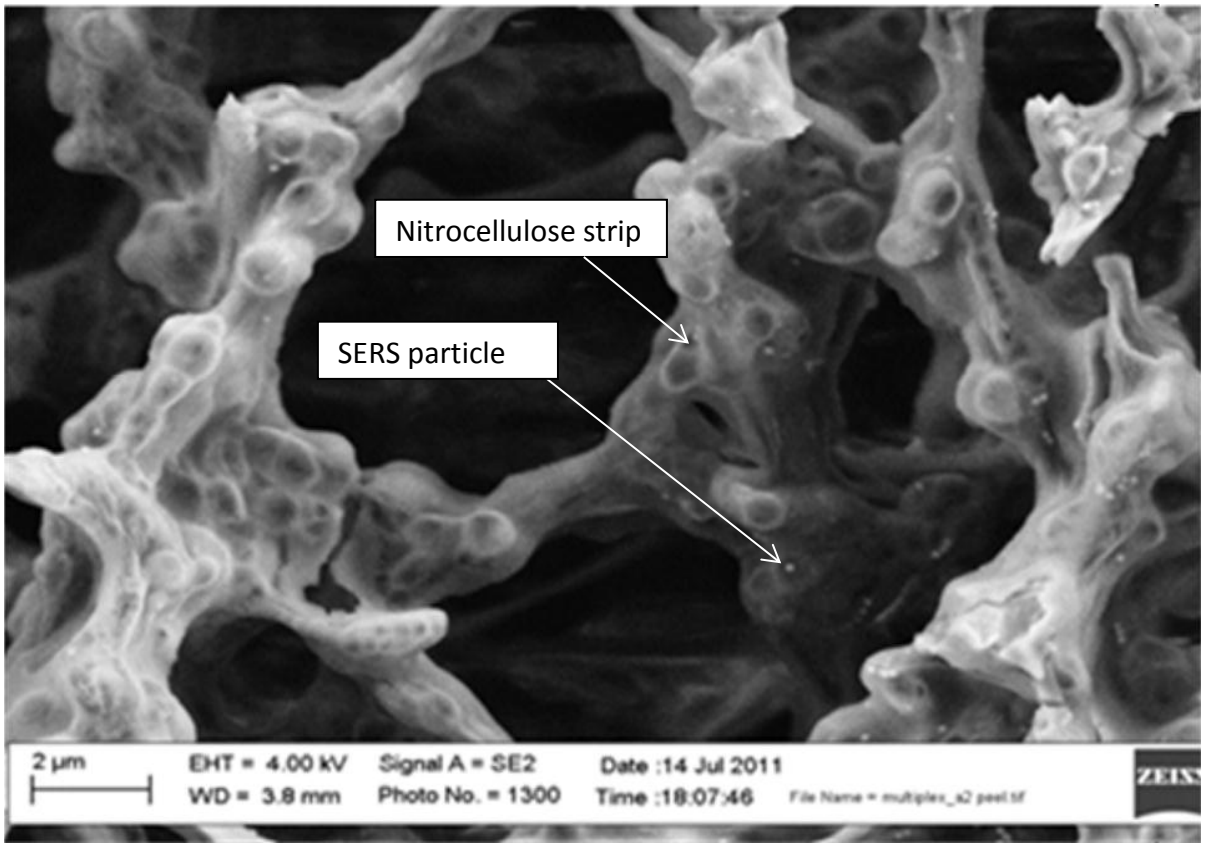


Figure 5.6: Test line image of nitrocellulose strip with all three SERS particles in highest concentration (CRP 5000 ng/ml, myoglobin 500 ng/ml and troponin 50 ng/ml).
Reprinted with permission from Noble, J., Attree, S., Horgan, A., Knight, A., Kumarswami, N,, Porter, R., and Worsley, G. (2012). Optical scattering artifacts observed in the development of multiplexed surface enhanced Raman spectroscopy nanotag immunoassays. *Anal. Chem.* **84**, 8246-8252 Supplementary information.

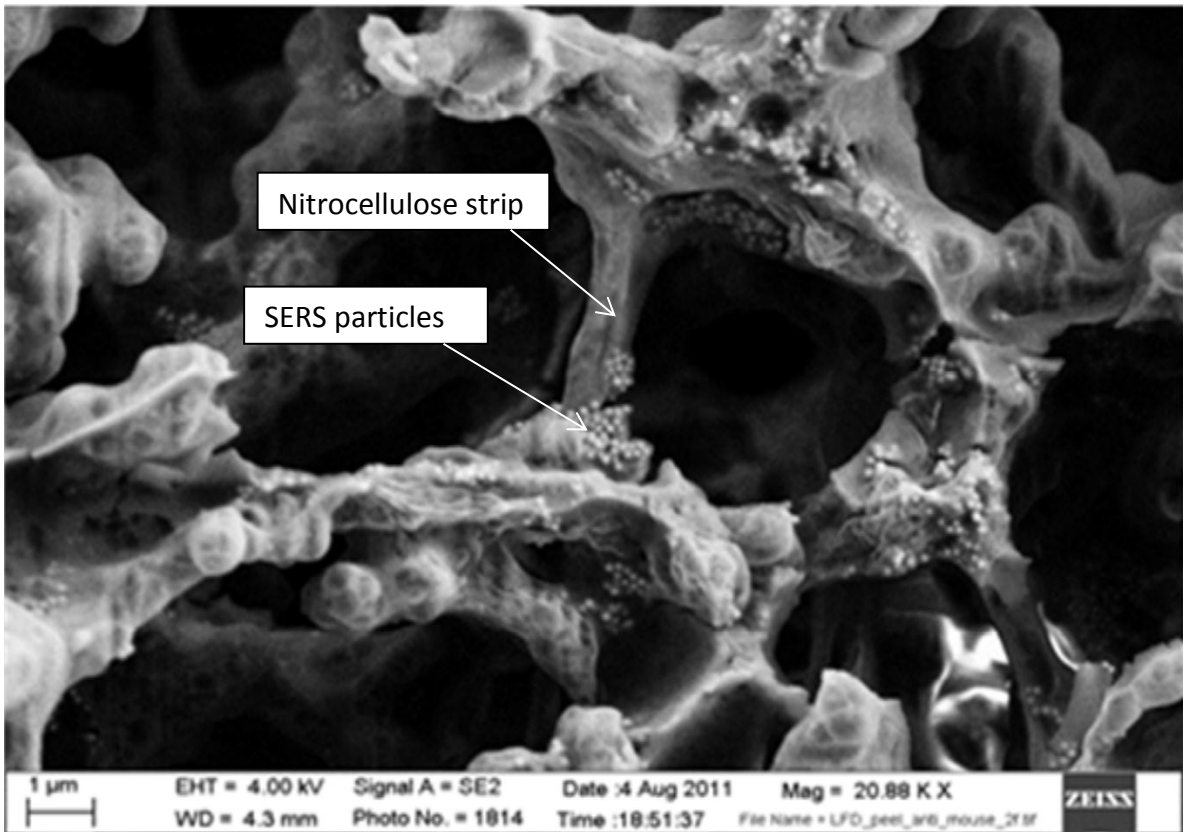


Figure 5.7: Test line image of nitrocellulose strip with 470 SERS particles and anti-mouse antibody with three time higher compare to the Figure 5.6.
Reprinted with permission from Noble, J., Attree, S., Horgan, A., Knight, A., Kumarswami, N,, Porter, R., and Worsley, G. (2012). Optical scattering artifacts observed in the development of multiplexed surface enhanced Raman spectroscopy nanotag immunoassays. *Anal. Chem.* **84**, 8246-8252 Supplementary information.

5.3 Optical interference

The third possibility considered was the effect of light scattering by SERS particles at or near the surface of the test line of assay strip that is the closest to illumination source. In this case there are many SERS particles (all three analytes at high concentration) at the surface, so the amount of scattered light will be large compared to the situation when few particles (only one analyte is at high concentration in the mixture) are at or near the surface. Consequently there is the possibility that particles in the bulk of the assay strip receive less incident photons than expected. This would result in low Raman signal. Figure 5.8 where the presence of multiple analytes all at a high concentration in the test line, increases the absorption and scattering of the incident and reflected light and resulting in lower optical penetration into the lateral flow strip and therefore decreased the signal.

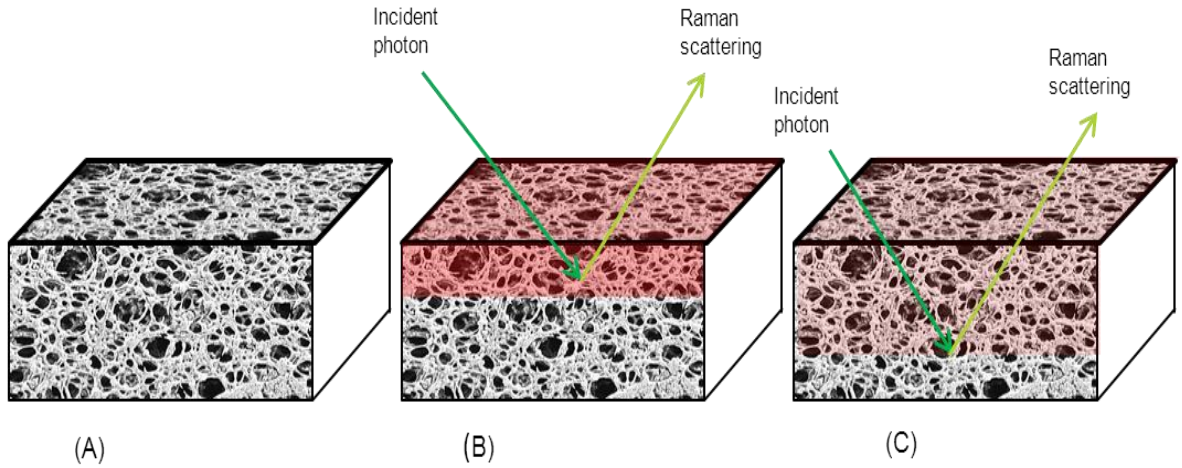


Figure 5.8: Schematic demonstrating the scattering hypothesis. (A) Test line in a nitrocellulose strip, expanded right. (B) Multiplex assay strip-where all three analytes are present at high concentration, On right (C) Multiplex assay strip -incident photon and scattering light when only one analyte presence in high concentration.

This optical scattering artefact is observed in the Multiplexed SERS assay, when all three analytes are present in high concentrations and published (Noble *et al.,* 2012). To investigate this, 470 and 421 particles were deposited on a lateral flow strip and loss of linearity was detected consistent to the increased concentration of the second particles.

Figure 5.9 Shows that the addition of the other particles to the assay resulted in the decrease in the measured intensity of the first particle.

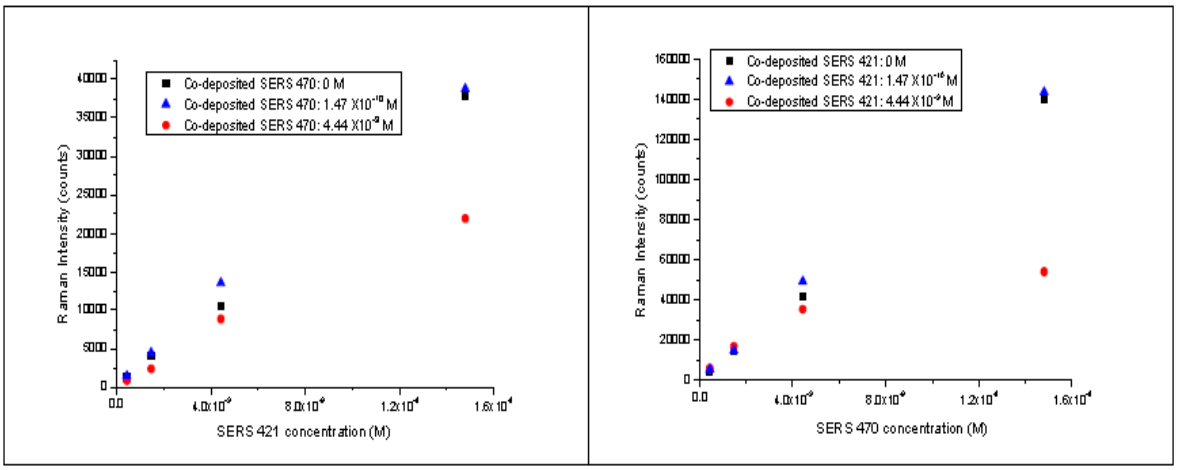
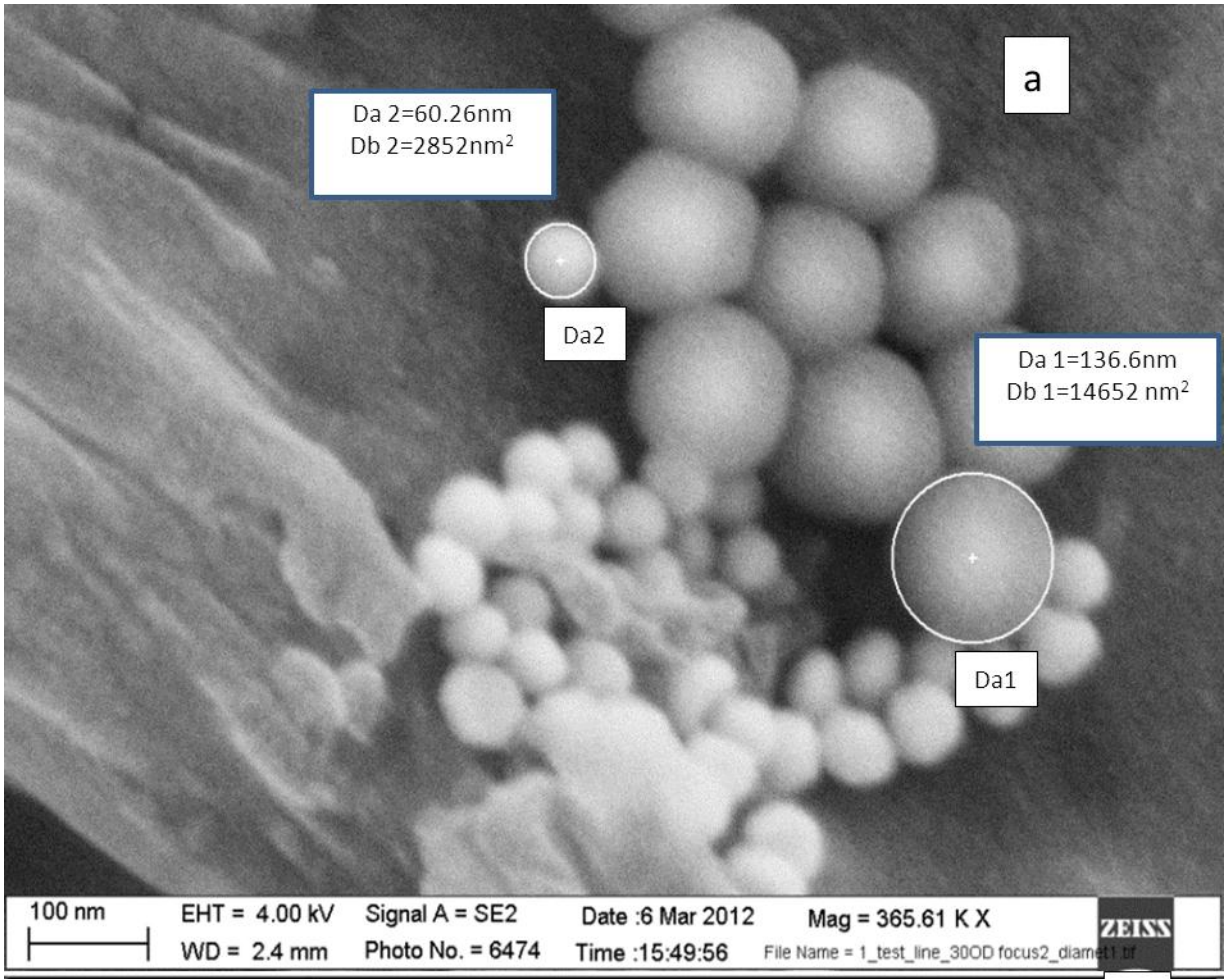


Figure 5.9: (Left) 421 SERS particles spotted on nitrocellulose lateral flow strip and imaged. By increasing the co-deposition of 470 particles in the assay shows decrease in the Raman intensity of 421 particles. (Right) 470 SERS particles spotted on nitrocellulose lateral flow strip and imaged. By increasing the co-deposition of 421 particles in the assay shows decrease in the Raman intensity of 421 particles. Reprinted with permission from Noble, J., Attree, S., Horgan, A., Knight, A., Kumarswami, N., Porter, R., and Worsley, G. (2012). Optical scattering artifacts observed in the development of multiplexed surface enhanced Raman spectroscopy nanotag immunoassays. *Anal. Chem.* **84**, 8246-8252 Supplementary information. Copyright 2012 American Chemical Society.

The above result suggested that the optical density of the SERS particles in the assay is causing the interference. For enhanced understanding similar size (at 785nm) of gold particles titrated with the fix concentration of SERS 470 and deposited on lateral flow strip. SEM was used to count the particles in each test line. Concentrations of gold and SERS were measured by absorbance at 520 nm and calculation of optical properties of SERS particles was performed using Mie theory calculate software. (Charamisinau *et al.,* 2004; Jain *et al.,* 2006). Table 5.2 shows that the absorption efficiencies of the particles are relatively low (~0.05 and 0.11) but the scattering efficiencies are very high (1.05 and 1.22). Figure 5.10 (a) shows that the SERS and gold particles are easily differentiated in the assay using SEM image and (b) shows the density of the SERS particle/μm^2 of the test line. This was

performed to ensure that the addition of the gold particles in to the assay did not alter or disturb the density of SERS particles. These strips were analysed by Raman reader and plotted as figure 5.11. This suggests that the Raman signal of the SERS particles decreases as the addition of gold particles increases in the assay. Results are consistent as observed in Figure 5.9 (Noble *et al.,* 2012).



b

Au colloid concentration	SERS nanotags (particle number/μm^2)		
	Mean	Std	Median
0	2.93	2.63	2.03
4.62×10^{-9} M	2.41	2.54	1.67
9.24×10^{-9} M	3.36	2.85	2.15
1.85×10^{-8} M	3.14	2.06	2.37

Figure 5.10: (a) An SEM image of the test line with mixture of gold and SERS particles. (b) The density of SERS particles on the test line with gold particles concentration. The data represents the mean, slandered deviation and median of twenty images of the test line. Reprinted with permission from Noble, J., Attree, S., Horgan, A., Knight, A., Kumarswami, N,, Porter, R., and Worsley, G. (2012). Optical scattering artifacts observed in the development of multiplexed surface enhanced Raman spectroscopy nanotag immunoassays. *Anal. Chem*. **84**, 8246-8252 Supplementary information. Copyright 2012 American Chemical Society

Particle type	Wavelength (nm)	Core Radius (nm)	Shell Radius (nm)	Q_{sca}	Q_{abs}	Q_{ext}	ε ($M \cdot cm^{-1}$)
Gold	785	60.0	—	1.22853	0.113281	1.34181	5.26×10^8
SERS		34.7	69.0	1.05473	0.051897	1.10663	2.30×10^9
	560			0.88915	0.404861	1.29401	2.68×10^9

Table 5.2: Scattering, absorption and extinction efficiencies, and molar decadic extinction coefficients, for core-shell and naked gold particles.
Reprinted with permission from Noble, J., Attree, S., Horgan, A., Knight, A., Kumarswami, N,, Porter, R., and Worsley, G. (2012). Optical scattering artifacts observed in the development of multiplexed surface enhanced Raman spectroscopy nanotag immunoassays. *Anal. Chem.* **84**, 8246-8252 Supplementary information. Copyright 2012

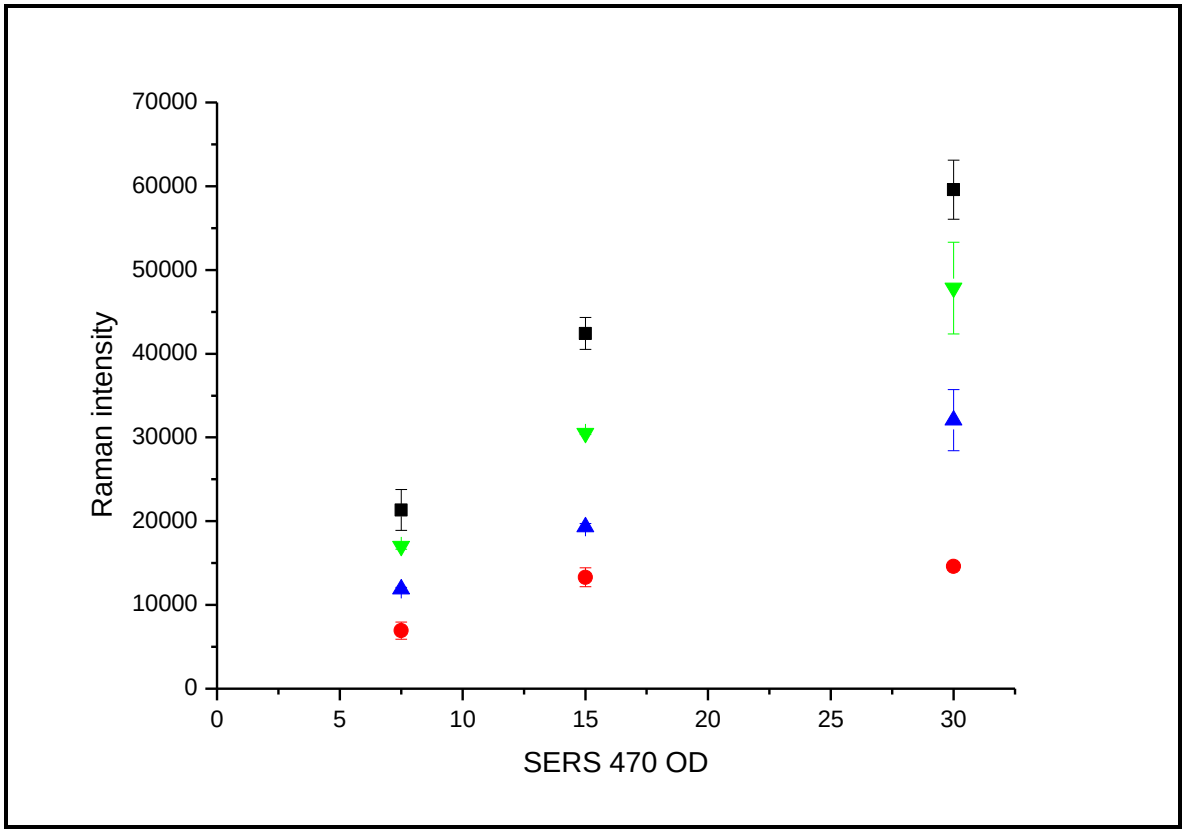


Figure 5.11: Titrations of SERS particles and Au colloids onto nitrocellulose.
■ Co-deposited Au colloids: 0 OD
▼ Co-deposited Au colloids: 7.5 OD
▲ Co-deposited Au colloids: 15 OD
● Co-deposited Au colloids: 30 OD
Reprinted with permission from Noble, J., Attree, S., Horgan, A., Knight, A., Kumarswami, N,, Porter, R., and Worsley, G. (2012). Optical scattering artifacts observed in the development of multiplexed surface enhanced Raman spectroscopy nanotag immunoassays. *Anal. Chem.* **84**, 8246-8252 Supplementary information. Copyright 2012 American Chemical Society.

5.4 Discussion

The objective of this chapter was to investigate the cause of the interference in multiplex assay observed in chapter 4. Three hypotheses were tested to identify the source of interference. Their outcomes are discussed below:

(1) Non-specific interactions between co-analytes and antibodies (Section 5.1):

As described in section 5.1, (A) cross- reaction between capture antibody of target analyte and detection antibody of co-analytes in absence of co-analyte; and (B) cross- reaction between capture antibody of target analyte and detection antibody of co-analytes in the presence of co-analyte were analysed for troponin, myoglobin and CRP. Analysis of the twelve possibilities showed no significant cross-activity between co-analytes or their detection antibodies with the targeted capture antibody. However, other cross-reaction (equation C and D) were overlooked and could have been investigated (Table 5.1.) Thus, the outcome of the first hypothesis didn't show any significant evidence of immunological interference in the assay.

(2) "Steric hindrance/crowding" between the SERS nanoparticles on test line (Section 5.2):

A two-run assay with troponin was designed to test the possibility of steric hindrance. A small effect of the CRP and myoglobin interferences on troponin intensity was observed. Troponin intensity was found to be inhibited when it was run in a mixture with CRP or myoglobin. Correspondingly, troponin intensity was slightly inhibited after running CRP or myoglobin assay (Figure 5.3 and Figure 5.4). This results hinted clogging on the strip, but the SEM images of multiplex strip with highest concentrations ((troponin 50 μg/L, myoglobin 500 μg/L and CRP 50 mg/L) gave evidence of no clogging on the strip and adequate space between the particles was observed on the test line. Additionally, an anti-mouse assay strip

with three time higher number of particles was compared with the multiplex strip. To summarise, this investigation did not conclude that the steric hindrance was the cause of interference in the assay.

(3) Optical Interference (Section 5.3):

To understand the issue of interference in multiplex assay, a simpler SERS duplex assay was examined using 470 and 421 particles. Raman intensity of 470 particles was found to decrease with increase in co-deposition of 421 particles and vice-versa. Optical interference effect was also investigated using 470 particles (diameter ~ 140 nm) as a model and titrating naked gold particles (diameter ~ 60 nm) in the assay. The Raman intensity of the SERS particles was measured in the presence of different concentration of gold-colloid. The results showed that Raman intensity from the SERS particles decreased as the concentration of gold particles was increased, which is similar to the effect observed in the multiplex assay. Nanoparticle concentration was obtained via absorbance measurements at 520 nm. Very high scattering efficiencies (1.05 for SERS and 1.22 for gold) and very low absorbance efficiencies were observed (0.05 for SERS and 0.11 for gold) from the calculation of optical properties using the Mie theory. Furthermore, the plot of SERS intensity of the particles vs. their density (measured from SEM images) confirmed the decrease in Raman intensity as with the increase in number of particles in the assay (Figure 5.11). Therefore, the optical interference effect suggests the use of a reduced number of particles to obtain a quantitative assay. However, a possible difficulty in reducing SERS particles in the assay is that it would also limit the detection range of troponin and myoglobin. As a result a sample with low concentration of biomarkers (i.e. troponin ~ 1 ng/ml or myoglobin ~ 6 mg/ml) may not be detected easily using the reduced number of particles. To eliminate the interferences a quantitative assay for TNFα or IL6 (inflammation markers) with SERS particles was

prepared and directly compared with the fluorescence assay in the attached publication (Noble *et al*., 2012; Worsley, *et al*., 2012). These assays have used less number of SERS particles on lateral flow strips and hence found minimal interferences. A direct comparison with fluorescence assay demonstrated that that a semi-quantitative assay with SERS is possible for wound healing markers with minimum interferences with detection range similar to the fluorescence assay.

In summary, this chapter have tested all possible sources of interference in the multiplex assay. Investigation revealed that the optical density of the SERS particles was the main cause of the interference in the measurements. Therefore, to obtain a quantitative assay by less number of particles should be used in measurements to eliminate the interference.

CHAPTER 6: General Discussion

6.1 Background and modelling of the assay

Previous studies have demonstrated the significance of multiple analytes detection in point of care (POC) and various established technologies have tried to address this diagnostic requirement (Woo *et al.,* 2009; Worsley *et al*., 2012). However, there have been limitations like cost, restriction of detection, interference or necessity of multiple stages for testing complex mixtures. This study has been undertaken to explore the potential of SERS particles to enable multiplexed immunoassay in lateral flow format (Figure 6.1).

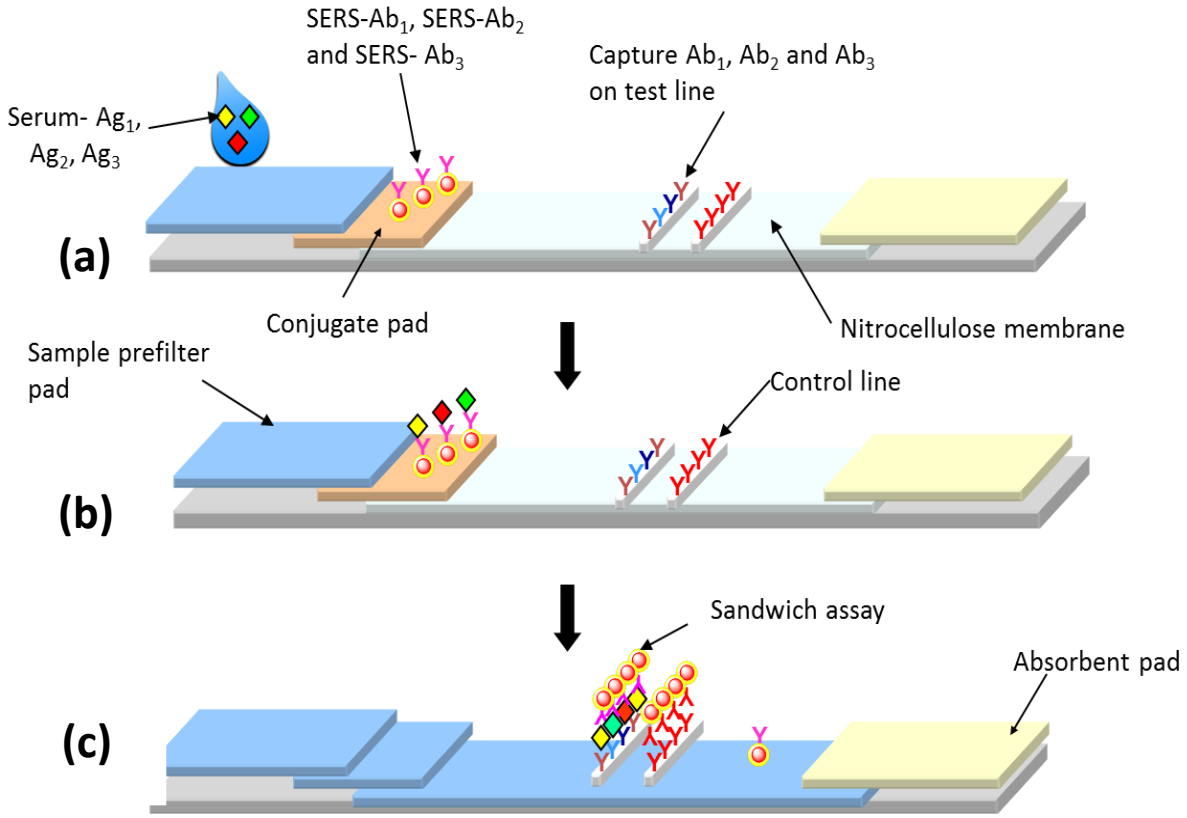


Figure 6.1: Schematic of multiplex assay on a single test line. (a) Arrangement of lateral flow assay incorporating a single test line with capture antibodies (Ab) for each of three target antigens (Ag) and respective SERS conjugate (SERS-Ab) preloaded on conjugate pad. (b) analytes (Ag) pass through the conjugation pad and bind with their respective SERS conjugate. (c) Sample (Ag-SERS-Ab complex) passes onto the test line and makes a sandwich assay. Unbound sample with the excess SERS particles moves onto the control line to bind with control antibody and rest of the fluid passes toward the absorbance pad.
[Ag1]: Troponin; [Ag2]: myoglobin; [Ag3]: CRP.
[SERS-Ab1]: 470-M19C7; [SERS-Ab2]: 440-7C3; [SERS-Ab3]: 421-C6.
[Capture Ab1]: MF4+560; [Capture Ab2]: 4E2; [Capture Ab3]: C2.

The SERS particles (420, 440, 470 and 421) utilised in this research were developed by Oxonica LTD (United Kingdom). To establish the concept of multiplex detection, troponin, myoglobin and CRP were selected from an extensive background investigation of biomarkers having the utility in myocardial Infraction detection. To read the signal from the SERS particles, a Raman System R-3000 QE (Ocean Optics, Dunedin, USA) was purchased and modified to work with a lateral flow strip holder and stage. Furthermore, a technique (Y-axis scan with 37 mm/s speed) was developed to read the real-time quantitative and qualitative signal analysis from the SERS particles on the lateral flow strip (Section 3.3).

First, Raman spectra of the individual commercially obtained SERS particles were obtained using the Raman reader. Then the Raman spectra were obtained from a mixture of the SERS particles. After the analysis of the individual and mixed Raman spectra, three SERS particles were chosen (421, 440 and 470), based on their well-separated unique Raman peaks (921 cm^{-1}, 1335 cm^{-1} and 1159 cm^{-1}) to perform the multiplex assay. This was aimed to avoid confusion in distinguishing unique features when analysing a diversity of samples. Additionally, a relationship between the Raman signal intensity and the concentration of the SERS particles in aqueous solution was established using the Raman reader. This relationship was found to be linear (Sections 3.1 and 3.2).

From the literature review of this study, techniques like UV-Vis, DLS and lateral flow assay were identified for the particles' characterisation. The examinations of each particle before and after conjugation by DLS and UV-Vis spectroscopy confirmed their protein attachment on surface, correct size of particles and the lack of aggregation. These results also confirmed the stability of the particles after antibody labelling. The antibody labelling on selected SERS particles (421, 440 and 470) were carried out under different buffer and pH, to select the ideal conditions for the assay. The ideal condition for the preparation of conjugates were found to be: (1) Troponin-470 with 300:1 (M19C7: sulfo SMCC) ratio in 50mM sodium borate pH 7.2; (2) Myoglobin-440 with 60:1 (7C3: sulfo SMCC) ratio in 50mM sodium borate pH 6.5 and (3) CRP-421 with 35:1 (C6: sulfo SMCC) ratio in 50mM sodium borate pH 7.2. Using these conditions a stable single lateral flow assays were performed first in buffer and later in serum, across the range of their pathological values. These results provided a deep understanding of each candidate's individual behaviour and confirmed their suitability to perform multiplex lateral flow assay (chapter 3).

6.2 Multiplex assay

The principal aim in this study was to develop multiplexing biosensor platform. A first multiplexing assay examination was performed using the similar condition for preparing and running as they were used in individual analysis. The initial multiplexing assay results showed a poor performance of troponin and myoglobin as the Raman signal intensity didn't change significantly with a change in concentration. The intensity of each analyte in the multiplexing assay was lower than their individual lateral flow analysis. To improve the Raman signal intensity from the analytes an increased SERS particles-antibody was carried out. This enabled each analyte to be successfully detected across their pathological values using the Raman reader.
In summary, the goal of this study was to demonstrate the proof of concept multiplex assay. This was shown using a well-established cardiac model system. Each selected analyte and their measurable ranges in final single and multiplex assay are summarised here in Table 6.1. Their IC_{50} values have been calculated by sigmoidal logistic fit.

Analytes	**Single analyte assay**		**Multiplex assay**	**Pathological range**
	Buffer	**Serum**	**serum**	
Troponin(ng/ml)	200-1.4	100-0.3	43-0.06	30-0.05
Measured IC $_{50}$	19	15.8	2.3	
Myoglobin(ng/ml)	400-6	415-3	533-0.5	500-5
Measured IC $_{50}$	13.9	19.3	2.2	
CRP(ng/ml)	4000-63	4000-32	5000-7	3000-100
Measured IC $_{50}$	0.57	317	700	

Table 6.1: Summary of each analyte measured ranges and their calculated IC_{50} values compared to their dynamic ranges in single and multiplex assay.

However, interference issues were observed during the further quantitative analysis of the multiplexing assay while using high, medium and low concentration of analyte. The results showed that an individual analyte's Raman intensity was inhibited by the addition of co-analytes in high concentration. Therefore, individual analyte interference assays were investigated separately in the absence or presence of high and low concentrations of co- analytes. Thus, the interference challenge was identified at this stage of the study (Chapter 4).

6.3 Investigation of interference in the multiplex assay

The origin of the interference issue was also explored in this study eliminate the inhibition of Raman signal in the multiplexing assay. Three possible sources of interference were investigated in chapter 5: (1) Non-specific interaction assay – Interaction between the target antibody and co-analytes; (2) Steric - effect (crowding effect); (3) Optical interference.

The output of the non-specific interaction assay test indicated no possibility of cross activity between antibodies and co-analytes in the assay. Investigation of the steric effect using two-run assay suggested that the crowding of particles at high concentration might the cause of the inhibition of Raman signal because troponin signal was affected after CRP or myglobin assay. But SEM images confirmed that there was no crowding on the test line of the multiplexed assay. Hence the investigation of the steric-effect did not confirm the cause of the interferences. Finally, the investigation of the optical interference was carried out in detail. First, the titration of the SERS particles and naked gold particles assay of same size confirmed that the Raman intensity of SERS particles was decreasing with the increase in gold particle concentration in the assay. This outcome was similar to the observation of the inhibition of Raman intensity observed in the previous multiplexing assay. Calculated optical property outcomes of the SERS and gold particles showed that the scattering efficiencies were very high and absorbance efficiencies were very low. Hence, the observation of the decrease in Raman intensity of the target analyte was attributed to increase in the Rayleigh scattering of laser light from the high number of particles deposited on the test line. This last interference was analysed within the team at NPL and published in *Analytical Chemistry* (Noble *et al.,* 2012). This examination suggested reducing the number of particles used in the assay to eliminate the interference. However, the disadvantage of reducing particles concentration in the assay would potentially limit the assay sensitivity. Therefore, the SERS assay was demonstrated with reduced particles using wound healing biomarkers (TNFα and IL6) showed the minimal interference in the assay.

In conclusion, this study has demonstrated that the main advantage of using SERS particles in lateral flow assays is their ability to multiplex. It has been shown that semi-quantitative and qualitative multiplexing assays can be performed with a negligible interference using a reduced number of particles in the assay. However, a limitation of using SERS particles is that the high light scattering from gold nanoparticles limits light penetration into the test line. Therefore, less number of particles are recommended to be used in the multiplex assays.

6.4 Future research

A number of assays can be conducted in the future research to improve the technique of multiplex immunoassay that has been proposed and demonstrated in this study. Four aspects of current methodology seem particularly worthy of a deeper look in the future:

1. Examination of the cross-reaction modes C and D that have been overlooked in this work (section 5.1). In the presence and absence of a specific analyte, the cross-reaction of the capture antibody and detection antibody with a co-analyte needs to be further tested on a lateral flow strip. Therefore, twelve possibilities need to be tested as described in section 5.1. Even though, the probability of the cross reaction is low, but if any cross-activity is found then further investigation could be carried out to eliminate the immunological issues. This further work will support the preliminary investigation of the present study and confirm the quality of the materials used in current assay.
2. Investigation of the selected cardiac biomarkers with a reduced number of SERS particles (roughly two-four times less) could be conducted. This investigation would be expected to eliminate the interferences from the current set-up, which is crucial to demonstrate a successful model within the dynamic range of the biomarkers. By reducing the number of SERS particles in the assay the limit of detection might arise for the lowest concentration of biomarkers (troponin: 0.05 ng/ml and myoglobin: 5 ng/ml). The sensitivity issue could be resolved by increasing the capture antibody concentration (MF4+560, 4E2) on the test line to improve the binding efficiency of the conjugate.
3. Effect of high or low concentration of each capture antibody on test line could be analysed in the future. This examination would demonstrate the maximum binding efficiency of each analyte on the strip and hence would lead to the improvement of the existing model system by increasing the detection range of the analyte. It would also confirm the maximum or minimum requirement of the capture antibody in a mixture to obtain an improved Raman signal from the test line.
4. To commercially launch a point of care (POC) biosensor platform with SERS particles a few quality control tests of the lateral flow strip need to be performed in addition to above analysis. This will include:

- Examination of various distances of the test line from the bottom of the strip. Currently, the standard distance of the test line is 5 mm from the start of the strip. Therefore, testing of 4 mm (closer to the bottom) to 6 mm (far from the bottom) distance from the start of the lateral flow strip would be required to identify the effect of distance.
- Testing of various drying times (10 min, 30 min and longer) for the strip after running an assay needs to be performed. Both of these quality control tests would help to obtain a higher intensity of the Raman signal.

5. Furthermore, SERS particles can be certainly utilised to detect a range of analytes in lateral flow assay in POC, for example, wound healing markers (TNFα, IL6 and MMP9) and kidney function biomarkers (serum creatinine, β-Trace protein, cystatin C). This will demonstrate the versatility of SERS particles for diagnosis of diverse range of diseases in a multiplex format. The attached publication has found that the dynamic range of inflammation marker detected by SERS particles is similar to fluorescence detection in a duplex format (Noble *et al*., 2012). However, it would be valuable to compare the SERS particles and fluorescence labelling using cardiac biomarkers in a multiplex format. This can show the advantages of SERS particles over the fluorescence labelling in diagnostic research.
6. The long-term direction of current concept would be to construct a platform of using more (6-9) SERS particles for multiple (6-9) analyte detection by developing a model of three test lines on the single lateral flow strip. Currently, there are hardly any studies that have addressed or demonstrated this concept. Therefore, this can be a very inspirational step in the lateral flow domain, even though many challenges are expected to appear in the assay. This model will require two to three capture antibodies on one test-line. Hence, 6 - 9 analytes can be detected using three test lines (more test line may not fit in a small lateral flow strip). For the success of such a model, various steps of analysis would be required for this examination, for example, choosing correct biomarker range (with a similar dynamic range), analysing combinations of SERS particles, fabrication of three test line with sufficient space to avoid interferences, examining capture antibody and detection antibody loading for each analyte, investigation of interference as the complexity of assay will increase

etc. The success of this concept could launch the routine use of SERS particles in diagnostic analyses and herald a novel area of POCT development.

REFERENCES

Aubin-Tam, M. and Hamad, K. (2008). Structure and function of nanoparticle–protein conjugates. *Biomed. Mater.* **3,** 034001.

Ahammad, A. J. S., Choi, Y., Koh, K., Kim, J., Lee, J. and Lee, M. (2011). Electrochemical detection of cardiac biomarker troponin I at gold nanoparticle-modified ITO electrode by using open circuit potential. *Int. J. Electrochem. Sci.* **6,** 1906-1916.

Algarra, M., Gomes, D. and Esteves da Silva, J. C. G. (2013). Current analytical strategies for C-reactive protein quantification in blood. *Clin. Chim. Acta* **415,** 1-9.

Baena, J. R. and Lendl, B. (2004). Raman spectroscopy in chemical bioanalysis. Curr. Opin. Chem*. Biol.* **8,** 534-539.

Bishnoi, S. W., Lin, Y., Tibudan, M., Huang, Y., Nakaema, M., Swarup, V. and Keiderling, T. A. (2011). SERS biodetection using Gold–Silica nanoshells and nitrocellulose membranes. *Anal. Chem.* **83,** 4053-4060.

Brown, L. O. and Doorn, S. K. (2008). A controlled and reproducible pathway to dye-tagged, encapsulated silver nanoparticles as substrates for SERS multiplexing. *Langmuir* **24,** 2277-2280.

Charamisinau, I., Happawana, G., Evans, G., Rosen, A., Hsi, R. and Bour, D. (2005). Semiconductor laser insert with uniform illumination for use in photodynamic therapy. *Appl. Opt.* **44,** 5055-5068.

Chun, P. (2009). Colloidal gold and other labels for lateral flow immunoassays. In *Lateral Flow Immunoassay* (ed. R. Wong and H. Tse), pp. 1-19: Humana Press.

Clearfield, M. B. (2005). C-reactive protein: A new risk assessment tool for cardiovascular disease. *J. Am. Osteoph. Assoc.* **105,** 409-416.

Collinson, P. (2000). Cardiac marker testing. *Ann. Clin. Biochem.* **37,** 733-734.

a) Davies, C. (2005). Concepts. In *The Immunoassay Handbook* (ed. D. Wild), pp. 103-135. Amsterdam: Elsevier.
b) Davies, C. (2005). Introduction to immunoassay principles. In *The Immunoassay Handbook* (ed. D. Wild), pp. 3-40. Amsterdam: Elsevier.

de Beer, F. C., Hind, C. R., Fox, K. M., Allan, R. M., Maseri, A. and Pepys, M. B. (1982). Measurement of serum C-reactive protein concentration in myocardial ischaemia and infarction. *Br. Heart J.* **47,** 239-243.

de Winter, R. J., Koster, R. W., Sturk, A. and Sanders, G. T. (1995). Value of myoglobin, troponin T, and CK-MB_{mass} in ruling out an acute myocardial infarction in the emergency room. *Circulation* **92,** 3401-3407.

Dehghan, A., Kardys, I., de Maat, M. P. M., Uitterlinden, A. G., Sijbrands, E. J. G., Bootsma, A. H., Stijnen, T., Hofman, A., Schram, M. T. and Witteman, J. C. M. (2007). Genetic variation, C-reactive protein levels, and incidence of diabetes. *Diabetes* **56,** 872-878.

Doering, W. E., Piotti, M. E., Natan, M. J. and Freeman, R. G. (2007). SERS as a foundation for nanoscale, optically detected biological labels. *Adv Mater* **19,** 3100-3108.

Huang, H., Liu, F., Huang, S., Yuan, S., Liao, B., Yi, S., Zeng, Y. and Chu, P. K. (2012). Sensitive and simultaneous detection of different disease markers using multiplexed gold nanorods. *Anal. Chim. Acta* **755,** 108-114.

HyTest Ltd. (2013). *High Sensitivity Human C-Reactive Protein (hsCRP).* Technical notes downloaded from http://www.hytest.fi/sites/default/files/hsCRP%20TechNotes.pdf (Accessed 26 March 2013)

Jain, P. K., Lee, K. S., El-Sayed, I. and El-Sayed, M. (2006). Calculated absorption and scattering properties of gold nanoparticles of different size, shape, and composition: applications in biological imaging and biomedicine. *J. Phys. Chem. B* **110,** 7238-7248.

John, H.T., LuongKeith B.M., Jeremy D.G. (2008). Biosensor technology: Technology push versus market pull. *Biotechnology Advances.**26**,* 492-500.

Kelly, K. L., Coronado, E., Zhao, L. L. and Schatz, G. C. (2003). The optical properties of metal nanoparticles: the influence of size, shape, and dielectric environment. *J. Phys. Chem. B* **107,** 668-677.

Kelly, K. L., Coronado, E., Zhao, L. L. and Schatz, G. C. (2003). The optical properties of metal nanoparticles: the influence of size, shape, and dielectric environment. *J. Phys. Chem. B* **107,** 668-677.

Kim, C., Ahn, J., Kim, J., Choi, J., Lim, K., Jung Park, T., Su Heo, N., Gu Lee, H., Kim, J. and Choi, Y. (2013). CRP detection from serum for chip-based point-of-care testing system. *Biosens. Bioelectron.* **41,** 322-327.

Kim, S. and Park, J. (2004). Development of a test strip reader for a lateral flow membrane-based immunochromatographic assay. *Biotechnol. Bioprocess. Eng.* **9,** 127-131.

Lagrand, W. K., Niessen, H. W. M., Wolbink, G., Jaspars, L. H., Visser, C. A., Verheugt, F. W. A., Meijer, C. J. L. M. and Hack, C. E. (1997). C-reactive protein colocalizes with complement in human hearts during acute myocardial infarction. *Circulation* **95,** 97-103.

Le Ru, E. C. and Etchegoin, P. G. (2009). *Principles of Surface-Enhanced Raman Spectroscopy*. Amsterdam: Elsevier.

Lewandrowski, K., Chen, A. and Januzzi, J. (2002). Cardiac markers for myocardial infarction: A brief review. *Pathol. Patterns Rev.* **118,** S93-S99.

Mauro, P., Franca, P. and Graziella, B. (2005). The sensitivity of cardiac markers: An evidence-based approach. *Clin. Chem. Lab. Med.* **37,** 1097-1106.

McCord, J., Nowak, R. M., McCullough, P. A., Foreback, C., Borzak, S., Tokarski, G., Tomlanovich, M. C., Jacobsen, G. and Weaver, W. D. (2001). Ninety-minute exclusion of acute myocardial infarction by use of quantitative point-of-care testing of myoglobin and troponin I. *Circulation* **104,** 1483-1488.

McDonnell, B., Hearty, S., Leonard, P. and O'Kennedy, R. (2009). Cardiac biomarkers and the case for point-of-care testing. *Clin. Biochem.* **42,** 549-561.

Nie, S. and Emory, S. R. (1997). Probing single molecules and single nanoparticles by surface-enhanced Raman scattering. *Science* **275,** 1102-1106.

Noble, J., Attree, S., Horgan, A., Knight, A., Kumarswami, N., Porter, R. and Worsley, G. (2012). Optical scattering artifacts observed in the development of multiplexed surface enhanced Raman spectroscopy nanotag immunoassays. *Anal. Chem.* **84,** 8246-8252.

O'Farrell, B. (2009). Evolution in lateral Flow–Based immunoassay systems. In *Lateral Flow Immunoassay* (ed. R. Wong and H. Tse), pp. 1-33. New York: Humana Press.

Osikowicz, G., Beggs, M., Brookhart, P., Caplan, D., Ching, S., Eck, P., Gordon, J., Richerson, R., Sampedro, S. and Stimpson, D. (1990). One-step chromatographic immunoassay for qualitative determination of choriogonadotropin in urine. *Clin. Chem.* **36,** 1586-1586.

Penttila, K., Koukkunenb, H., Halinenc, M. , Rantanena, T., Pyorala, K., Punnonena, K., Penttila, I. (2002) Myoglobin, creatine kinase MB isoforms and creatine kinase MB mass in early diagnosis of myocardial infarction in patients with acute chest pain. *Clin Biochem* **35(8),** 647-653**.**

Pepys, M. B. and Hirschfield, G. M. (2003). C-reactive protein: A critical update. *J. Clin. Invest.* **111,** 1805-1812.

Perry, S. V. (1979). The regulation of contractile activity in muscle. *Biochem. Soc. Trans.* **7,** 593-617.

Plebani, M. and Zaninotto, M. (1998). Diagnostic strategies using myoglobin measurement in myocardial infarction. *Clin. Chim. Acta* **272,** 69-77.

Stiles, P. L., Dieringer, J. A., Shah, N. C. and Van Duyne, R. P. (2008). Surface-enhanced Raman spectroscopy. *Annu. Rev. Anal. Chem.* **1,** 601-626.

Stuart, D. A., Haes, A. J., Yonzon, C. R., Hicks, E. M. and Van Duyne, R. P. (2005). Biological applications of localised surface plasmonic phenomenae. *Nanobiotechnology, IEE Proceedings* - **152,** 13-32.

Thermo Scientific Pierce. (2013). Instructions for SMCC and Sulfo SMCC downloaded from http://www.piercenet.com/instructions/2160581.pdf (Accessed 04 November 2013)

Thobhani, S., Attree, S., Boyd, R., Kumarswami, N., Noble, J., Szymanski, M. and Porter, R. A. (2010). Bioconjugation and characterisation of gold colloid-labelled proteins. *J. Immunol. Methods* **356,** 60-69.

Tran, N. K. and Kost, G. J. (2006). Worldwide point-of-care testing: Compendiums of POCT for mobile, emergency, critical, and primary care and of infectious diseases tests. *Point of Care* **5,** 84-92.

Ullman, E. F. (2005). Homogeneous immunoassays. In *The Immunoassay Handbook* (ed. D. Wild), pp. 212-232. Amsterdam: Elsevier.

Wang, G., Park, H., Lipert, R. J. and Porter, M. D. (2009). Mixed monolayers on gold nanoparticle labels for multiplexed surface-enhanced Raman scattering based immunoassays. *Anal. Chem.* **81,** 9643-9650.

Woo, M., Lee, S., Kim, G., Baek, J., Noh, M. S., Kim, J. E., Park, S. J., Minai-Tehrani, A., Park, S., Seo, Y. T. et al. (2009). Multiplex immunoassay using fluorescent-surface enhanced Raman spectroscopic dots for the detection of bronchioalveolar stem cells in murine lung. *Anal. Chem.* **81,** 1008-1015.

Worsley, G. J., Attree, S. L., Noble, J. E. and Horgan, A. M. (2012). Rapid duplex immunoassay for wound biomarkers at the point-of-care. *Biosens. Bioelectron.* **34,** 215-220.

Wu, A. H. B. (2006). A selected history and future of immunoassay development and applications in clinical chemistry. *Clin. Chim. Acta* **369,** 119-124.

Wu, L., Wang, Z., Zong, S., Huang, Z., Zhang, P. and Cui, Y. (2012). A SERS-based immunoassay with highly increased sensitivity using gold/silver core-shell nanorods. *Biosens. Bioelectron.* **38,** 94-99.

Zavaleta, C. L., Smith, B. R., Walton, I., Doering, W., Davis, G., Shojaei, B., Natan, M. J. and Gambhir, S. S. (2009). Multiplexed imaging of surface enhanced Raman scattering nanotags in living mice using noninvasive Raman spectroscopy. *Proc. Natl. Acad. Sci. USA* **106,** 13511-13516.

Zhu, J., Zou, N., Zhu, D., Wang, J., Jin, Q., Zhao, J. and Mao, H. (2011). Simultaneous detection of high-sensitivity cardiac troponin I and myoglobin by modified sandwich lateral flow immunoassay: Proof of principle. *Clin. Chem.* **57,** 1732-1738.

APPENDIX A: Publication

Optical Scattering Artifacts Observed in the Development of Multiplexed Surface Enhanced Raman Spectroscopy Nanotag Immunoassays

*J. Noble, S. Attree, A. Horgan, A. Knight, N. Kumarswami, R. Porter, and G. Worsley**
National Physical Laboratory, Hampton Road, Teddington, Middlesex, TW11 0LW United Kingdom

APPENDIX B: Poster presentation

MULTIPLEXING IMMUNOASSAYS USING SERS NANOPARTICLES

N. Kumarswami[1,2], A. Knight[1]*
1. National Physical Laboratory, Hampton Road, Teddington, TW11 0LW, UK
E-mail: Neelam.kumarswami@npl.co.uk
2. LIRANS Institute of Research in the Applied Natural Sciences, 250 Butterfield Great Marlings, Luton LU2 8DL.