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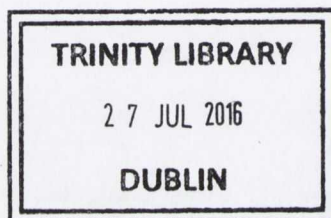
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Development of a nanowire-based assay for cancer biomarker detection in complex biological fluids



Submitted to the School of Clinical Medicine
for completion of doctoral studies
by
Omar Kazem Mahfoud

Under the supervision of Prof. Yuri Volkov
and co-supervision of Dr Adriele Prina-Mello



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Summary

The cost of delivering healthcare continues to increase to phenomenal proportions with lung cancer, breast cancer, colorectal cancer and prostate cancer accounting for the majority of the economic burden associated with cancer care in the EU. In this era of personalised medicine, medical treatments can now be selected and tailored depending on the specific biomarker profile of the patient. Multiplexed analysis of biomarkers is seen as a necessity for improving the future of biomarker-guided medicine. Nanomaterials are seen as one potential avenue for investigation in an attempt to reduce assay costs and increase multiplexing capabilities, and one of the key enabling technologies in the development of the next generation of clinical diagnostic assays.

In this thesis, the development of a nanomaterial platform for fluorescence-based flow cytometric detection of soluble cancer biomarkers in clinical samples is shown. Firstly, the platform was characterised and validated using a range of complimentary analytical approaches including confocal microscopy, atomic force microscopy, electron microscopy, flow cytometry, nanoparticle tracking analysis, spectroscopy and gel electrophoresis. Following this, the nanomaterial-based platform was used to demonstrate a proof-of-concept assay of ErbB2, EGFR and PSA cancer biomarkers in complex biological samples. Results show that the nanowire platform could produce comparable results to ELISA. Data analysis approaches for high-throughput nanowire interrogation in a flow cytometric system were also devised. Finally, the demonstration of a custom-made acquisition system for nanomaterial assays, with improved nanomaterial detection sensitivity over conventional flow cytometers was shown. It is hoped that this work can be further developed in future to deliver a nanomaterial platform for biomarker detection worthy of use in a clinical setting.

Acknowledgements

I would like to sincerely acknowledge the cooperation, guidance, patience, support and understanding afforded to me by Aoife, my parents, Hassan and Phyllis, my family, Yuri and friends and colleagues both past and present, during the fulfilment of the work contained henceforth. To name each individual would certainly cause unintentional omission, and all words of mine would fall short of expressing the true gratitude which I extend toward you all. I am much indebted to every one of you.

There are many lyrical analogies which spring to mind when I think of the past few years. For me, few capture the essence of our collective endeavours better than those immortal words sung by Luke Kelly, once upon a time;

“With our nets and gear we're faring
On the wild and wasteful ocean
It's out there on the deep
We harvest and reap our bread
As we hunt the bonny shoals of herring ...”

My wish is that I can be of some help to each of you in the same way that you are to me.

Sincerely,

Omar K Mahfoud

Publications List

Detection of ErbB2: nanotechnological solutions for clinical diagnostics. Omar K. Mahfoud, Tatsiana Y. Rakovich, Adriele Prina-Mello, Dania Movia, Frauke Alves and Yuri Volkov. RSC Advances, 2014, 4, pp 3422-3442. DOI: 10.1039/C3RA45401K, December 2013.

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Abbreviations

Ag	Silver
AP	Alkaline phosphatase
APTES	(3-Aminopropyl) triethoxysilane
Au	Gold
CISH	Chromogenic in situ hybridisation
CNT	Carbon nanotube
CoNi	Cobalt-Nickel
CTC	Circulating tumour cell
DNA	Deoxyribonucleic acid
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immuno-sorbent assay
EPR	Enhanced permeability and retention
ErbB2	Epidermal growth factor receptor 2
ER	Estrogen receptor
FACS	Fluorescence activated cell sorting
FDA	Food and Drug Administration
FISH	Fluorescent in situ hybridisation
HIM	Helium-ion Microscopy
HRP	Horseradish peroxidase
IgG	Immunoglobulin G
kHz	Kilohertz
IHC	Immunohistochemistry
LSPR	Localised surface plasmon resonance
μl	Microlitre
mL	Millilitre
mRNA	Messenger ribonucleic acid
NIR	Near-infrared
NM	Nanomaterial
NMRI	Nuclear magnetic resonance
NP	Nanoparticle
NTA	Nanosight Tracking Analysis
NW	Nanowire
PEG	Polyethylene glycol
PR	Progesterone receptor
QD	Quantum dot
RT-PCR	Real-time polymerase chain reaction

SERS	Surface enhanced Raman spectroscopy
SHG	Single harmonic generation
SPION	Superparamagnetic iron-oxide nanoparticle
SQUID	Superconducting quantum interference device
TEM	Transmission electron microscopy
TK	Tyrosine kinase
TM	Transmembrane
UV	Ultra violet
XRCT	X-ray computed tomography

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Chapter 1

Introduction and Literature Review

Detection of ErbB2: Nanotechnological solutions for clinical diagnostics

1.1 Introduction

1.1.1 Healthcare challenges, nanomaterials and thesis focus

The cost of delivering healthcare continues to increase to phenomenal proportions. Thanks to decades of scientific, technological, medical and fiscal contributions, we have reached a point where we can detect, diagnose, track and treat a vast array of illnesses with considerable success. This triumph however, does not come cheap. In the European Union alone, the economic burden of providing healthcare to cancer sufferers has reached a staggering €51.0 billion (Bn) in 2009, with lung cancer, breast cancer, colorectal cancer and prostate cancer accounting for the majority of the economic burden associated with cancer care (Luengo-Fernandez et al. 2013).

We are moving into the era of personalised medicine, where access to a new generation of genomic, proteomic and metabolomic assays, each capable of generating huge amounts of data from biological samples (through the measurement of specific biomarkers), has improved the depth of obtainable information from each patient and has increased the clinician's ability to make a well-informed medical decision. Treatments can now be selected and tailored depending on the specific biomarker profile of the patient. Companion diagnostics accompanying certain therapies are now also guiding patients and clinicians throughout the treatment course. Additionally, technological advances are helping to usher in personalised medicine. Computational capabilities have developed to such an extent that we can start to analyse and extract useful data from billions of scientific data points generated from biometric assays. Advances such as Deep Learning are allowing computers to begin to correlate data and identify clinically meaningful information that would simply be impossible for human clinicians to even begin to achieve. Medical device miniaturisation is ongoing and innovations in liquid handling, for example in flow cytometry and microfluidics, are driving a reduction in required biological sample volumes for assay purposes.

Although these advances will ultimately improve the quality of healthcare for the end user, they do add another layer of cost on top of the conventional healthcare budget. Another important facet influencing healthcare costs is the realisation that biomarker measurements are only really useful when viewed (a) over an extended time-course and (b) in the context of other biomarkers; meaning that the assay has to be carried out numerous times and several biomarkers need to be measured simultaneously (thus several mono-parametric assays must be used). At the moment, the prohibitive cost of a lot of assays prevents them from coming into widespread everyday use for the majority of the world's population. For now, biometric assessment using these powerful medical tools is currently reserved for those individuals (or

governments) who can afford to pay for them and are often beyond the reach of those who need them.

So how do we grapple with the challenge of reducing healthcare costs related to biomarker assay use whilst making improvements to the significant advances in molecular diagnostics achieved to date? It is necessary to reduce the cost of assays whilst increasing the number of different biomarkers that can be measured at one time (i.e. multiplexing). Nanomaterials are seen as one potential avenue for investigation in an attempt to reduce assay costs and increase multiplexing capabilities, and one of the key enabling technologies in the development of the next generation of clinical diagnostic assays.

1.1.1.1 Nanomaterials

A nanomaterial can be defined as any material where at least one of its dimensions can be measured on the nanometre (nm) scale, and are usually referred to as such when the properties of the nanoscale features contribute to the functional properties of material. The definition of a nanomaterial adopted by the EU commission (2011) states the following;

“A natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm - 100 nm. In specific cases and where warranted by concerns for the environment, health, safety or competitiveness the number size distribution threshold of 50 % may be replaced by a threshold between 1 and 50 %.”

The classification of nanoscale materials is based on the number of dimensions not on the nanoscale (<100nm). An in-depth overview of the categorisation of 0-, 1-, 2- and 3-dimensional nanomaterials (Illustrated in Table 3.1) is provided in (Ashby et al. 2009).

Nanomaterials carry a number of advantages for their potential integration into diagnostic kits. Their synthesis is relatively cheap and they can be produced on an industrial scale, their surfaces are easily functionalised with biologically active molecules, their dimensions, compositions and surface properties can be altered and they can be adapted to a range of detection modalities (e.g. fluorescence-based, magnetic, electronic) and biological media.

1.1.1.2 Thesis focus

This body of work forms part of the EU-funded FP7 NAMDIATREAM project, which is a multi-centre project which worked to develop innovative diagnostic devices for the early diagnosis of 3 common cancer types (lung, breast and prostate cancer). The presented work aims to lay the foundations for the development of a novel, cost-effective, nanomaterial-based system that could detect multiple cancer biomarkers in clinical samples. It involved the development of a nanowire-based assay platform for the detection of three well-established cancer biomarkers (ErbB2, EGFR and PSA), using ErbB2 as the archetypal biomarker for the initial proof-of-concept assay model. Firstly, the antibody pairs corresponding to the target cancer biomarker were chosen and validated. Secondly, the capture antibody was tethered to the nanowire surface to create a biologically active nanowire assay. The work relied on using a range of analytic approaches (including microscopy and flow cytometry) to assess the developing nanomaterial assay at various stages. The developed nanowire assay was tested for stability and functionality in detecting cancer biomarkers in a range of test scenarios, including spiked samples and real biological fluids. Finally, flow cytometry methods were developed which will enable the translation of nanowire use in flow cytometry-based molecular assays. It is hoped that this work will provide the basis for the development of a new assay platform that can help to deliver cost-effective, accessible, reliable healthcare diagnostics to a global population.

1.1.1.3 Thesis workflow synopsis graphic

The following graphic (Figure 1.1) synthesizes the workflow involved in developing a nanowire platform for detection of soluble cancer biomarkers. The validation of the nanoprobe as a proof-of-concept device comprises three main steps, namely; nanomaterial characterisation, functionalisation with antibody and detection of a cancer biomarker.

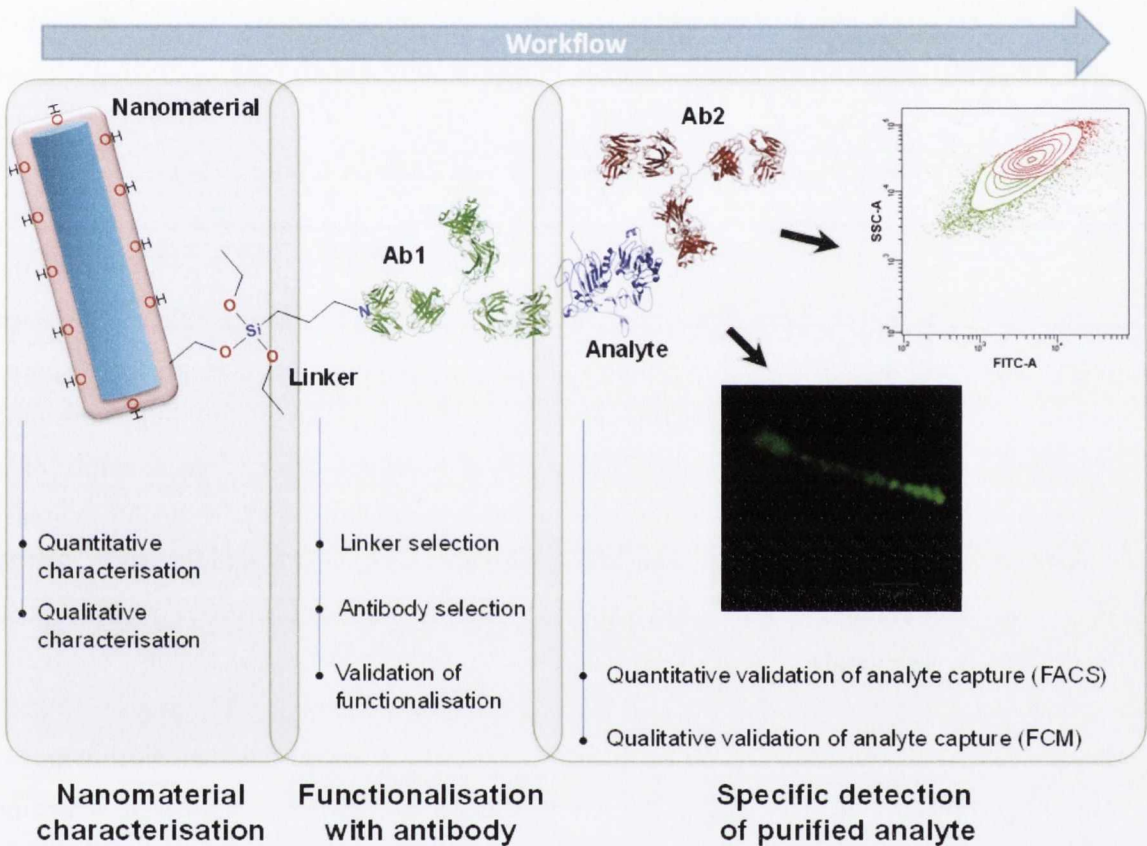


Figure 1.1. Schematic of development of nanowire assay for detection of soluble cancer biomarkers. Workflow: Nanomaterials (left hand side, light blue cylinder representing a nanowire) are characterised via a number of techniques (as explained in the materials and methods, page AA) including flow cytometry, nanoparticle tracking analysis, electron microscopy and atomic force microscopy. Target-specific green-fluorescent capture antibodies (Ab1) are conjugated to the nanomaterial surface via the linker molecule. (in this work APTES, see abbreviation and Section 2.2.5.2) Validation of functionalisation is achieved through use of fluorescent confocal microscopy, atomic force microscopy, Helium-ion microscopy, and flow cytometry (see Section 3.2.7). Finally, specific detection of purified analyte, using the target-specific fluorescent detection antibody (Ab2), is examined following incubation of the functional nanomaterial with the target analyte (see Section 4.2). Analyte capture is assessed quantitatively and qualitatively via flow cytometry and fluorescent confocal microscopy, respectively.

1.1.2 Cancer

Cancer can be broadly defined as uncontrolled cell division that results in abnormal cells that grow beyond their usual boundaries, and which may invade adjoining parts of the body and spread to other organs. The hallmarks of cancer include sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis (Hanahan & Weinberg 2011). Cancer is a leading cause of death worldwide, accounting for 8.2 million deaths in 2012, with lung, liver, stomach, colorectal and breast cancers being the most lethal. Lung cancer is the most common cancer worldwide (13% of all new cases diagnosed in 2012), followed by breast (11.9%), colorectal (9.7%) and prostate (7.9%) cancers (Stewart & Wild 2014).

Early detection and accurate diagnosis of cancers is seen as a major contributor to the prognostic outcome of cancer treatment. Over the past decade, huge emphasis has been placed on developing systems, procedures and policy to facilitate the development of cancer screening programs including breast, cervical, colorectal, prostate and lung screening programs (American Cancer Society 2015). Breast mammography is an example of a successful approach to cancer screening, with recent reports showing a 38% decline in breast cancer mortality among women who were screened with mammography (Paci 2012). However, mammography screening is costly and its cost-effectiveness is dependent on good health care infrastructure.

Progress in breast cancer treatment has probably advanced more quickly than treatments for any other cancer type. This is due in part to the above mentioned improved screening programs which facilitate early detection of the cancer, but is also heavily indebted to the scientific progress in identifying and stratifying breast cancer subtypes based on molecular markers. The development of companion diagnostic kits for breast cancer molecular subtypes, for use with a particular therapeutic product (such as the anti-ErbB2 monoclonal antibody therapy, Trastuzumab) has dramatically improved breast cancer survival rates in recent years (Perez et al. 2014). The ASCO (American Society of Clinical Oncology) has developed recommendations for molecular testing of breast cancer subtypes (Wolff et al. 2013) as well as evidence-based recommendations on the therapy guidelines for breast cancer depending solely on molecular subtype and disease staging (Giordano et al. 2014, Partridge et al. 2014, Van Poznak et al. 2015). This underlines the importance of detecting and accurately diagnosing breast cancer molecular subtypes in patients.

There are four major molecular subtypes in breast cancer which are classified based on the expression profiles of three receptors (Table 1.1) (Maryam et al. 2012, Wiechmann et al. 2009): luminal A (Estrogen Receptor (ER) and/or Progesterone Receptor (PR) positive, ErbB2 negative), luminal B (ER and/or PR positive, ErbB2 positive), basal-like (ER negative, PR negative, ErbB2 negative) and ErbB2 (ER negative, PR negative, ErbB2 positive).

Table 1.1 Breast cancer molecular subtypes. Breast cancer subtypes can be classified based on the expression patterns of estrogen receptor, progesterone receptor and ErbB2 receptor on the cancer cell.

Breast Cancer Molecular Subtype	Estrogen receptor (ER)	Progesterone receptor (PR)	ErbB2 receptor
Basal-like	—	—	—
Luminal A	+	+	—
Luminal B	+	+	+
ErbB2	—	—	+

Clinical studies have shown that patients with ErbB2-overexpressing primary tumours have a significantly greater potential to propagate nodal involvement, and extensive lymphovascular invasion, resulting in poor prognosis compared to individuals with Luminal A or basal expression of ErbB2 (Wiechmann et al. 2009). The activity of ErbB2 (i.e. the degree of receptor dimerisation with other growth factor receptors) in tumour tissue contributes to the aggressive behaviour of ErbB2 cancer subtypes (Amin et al. 2010). Thus, accurate assessment of ErbB2 in tumour cells holds significant diagnostic importance and may have therapeutic consequences.

1.1.3 Principle of ErbB2 function

Expression of the ErbB genes (of which four subtypes are known so far) yields formation of transmembrane glycoproteins with intrinsic tyrosine kinase activity and growth factor receptor function. ErbB2 is a receptor tyrosine kinase that belongs to the ErbB or EGFR (epidermal growth factor receptor) family (Figure 1.2). The ErbB2 receptor shows basal expression in many tissue types and is involved in normal tissue development and function, such as in the heart (Negro et al. 2006). Signalling through the ErbB2 receptor occurs following receptor dimerisation with the other ErbB family receptors or with another ErbB2 receptor itself. Such receptor dimerisation promotes cell growth and division, thus causing uncontrolled cell proliferation and hence tumour formation when overexpressed. Additionally, activation of the ErbB2 signalling pathways leads to an increase of tumour

metastasis-associated properties (such as invasion and angiogenesis) and induces therapeutic resistance via receptor-mediated anti-apoptotic signals (Yu & Hung 2000).

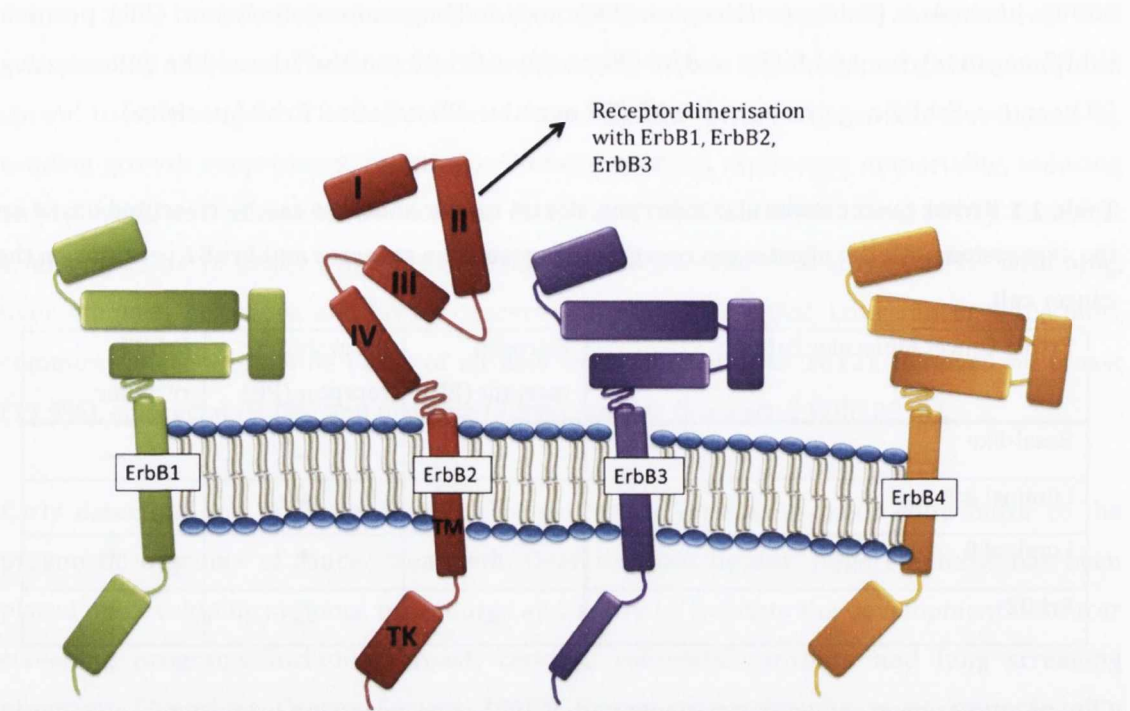


Figure 1.2. Schematic representation of the ErbB family, consisting of ErbB1 - ErbB4. The full length ErbB2 protein consists of 1255 amino acids comprising an extracellular domain (I-IV), transmembrane spanning region (TM) and an intracellular domain which holds tyrosine kinase (TK) activity. ErbB2 receptor dimerisation occurs through binding in the domain II. Receptor dimerisation results in initiation of downstream signalling events, facilitating processes such as cell proliferation, anti-apoptotic effects and angiogenesis. Figure adapted from Baselga & Swain (2009).

1.1.4 Clinical importance of ErbB2 detection: Focus on breast cancer

Since the mid-1980s, overexpression of the ErbB2 protein has been recognised as a feature of a malignant cancerous phenotype in breast cancer cell lines (Bargmann et al. 1986, Yamamoto et al. 1986) and has become one of the most widely investigated clinical indicators used to assess the severity of breast, ovarian, gastrointestinal and lung cancers (Dulak et al. 2012, Lanitis et al. 2012, Ugocsai et al. 2005, Wang et al. 2004) and guide targeted therapies (Murphy & Morris 2012). All of the current FDA-approved companion diagnostic kits for ErbB2 assessment can be used to guide Trastuzumab-based therapies in ErbB2-positive cancer patients (Table 1.2).

Table 1.2 FDA-approved companion diagnostic kits for Her2/ErbB2. [source: FDA.gov]

Treatment	Assay Name	Company
Trastuzumab	Pathvysion Her-2 DNA Probe FISH kit	Abbott Molecular Inc.
	Insite Her-2 kit	Biogenex Laboratories, Inc.
	Herceptest	Dako Denmark A/S
	HER2 FISH PharmDx Kit	Dako Denmark A/S
	Her2 CISH PharmDx Kit	Dako Denmark A/S
	Bond Oracle Her2 IHC system	Leica Biosystems
	Spot-Light Her2 CISH kit	Life technologies, Inc.
	Inform Her-2 FISH assay	Ventana Medical Systems
	Pathway anti-Her2 (4B5)	Ventana Medical Systems
	Inform Her-2 Dual ISH DNA assay	Ventana Medical Systems

Most of the focus around ErbB2 oncogenic function has been centred on its role in breast cancer. This is probably because breast cancer is by far the most commonly occurring form of cancer in women, accounting for 23% of total cancer incidences and contributing to nearly 14% of the cancer-related mortality (Cancer Genome Atlas 2012). The incidences of breast cancers are much higher in more developed regions (e.g. Western Europe) compared to less developed regions (e.g. Eastern Africa), whereas the mortality rate associated with breast cancer progression is similar between different world regions (Figure 1.2).

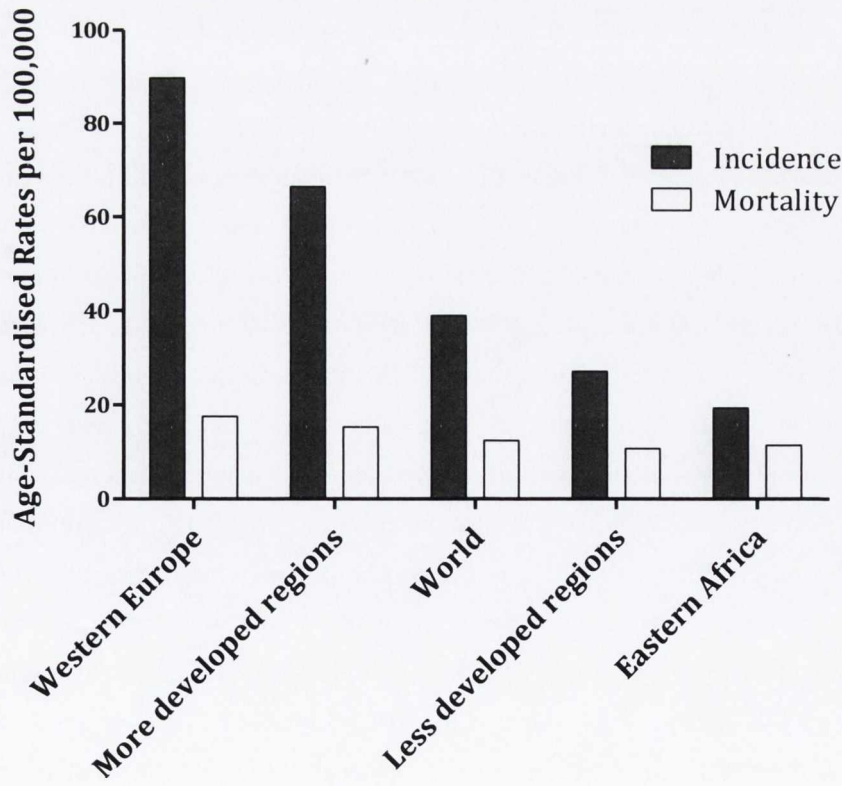


Figure 1.3 Incidence (black bars) and mortality (white bars) rates (age-standardised per 100,000 population) for breast cancer. Graph constructed from data retrieved from Globocan 2008 (globocan.iarc.fr).

1.1.5 Soluble ErbB2: Clinical importance in cancer diagnosis

Soluble ErbB2 protein exists in several forms due to a combination of alternate splicing and proteolytic processing (Figure 1.4). Alternate splicing results in the 100 kDa extracellular domain ErbB2 (comprising the first 633 amino acids of the full length protein), and the 68 kDa truncated Herstatin protein, which consist of the first 340 amino acids of the full length protein. Proteolytic processing of the full length protein also yields a 105/110 kDa soluble ErbB2 protein (Sasso et al. 2011).

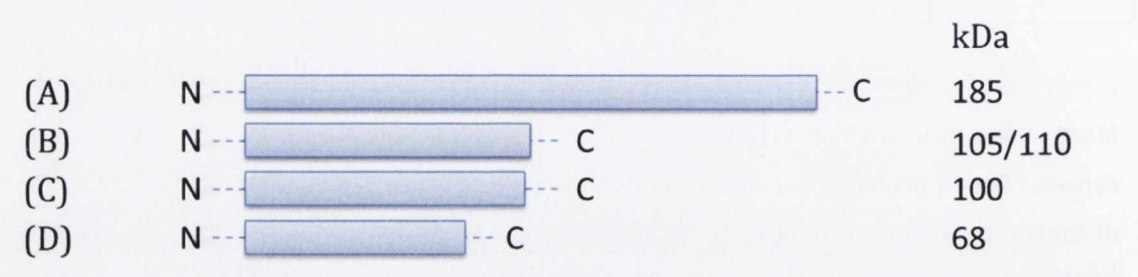


Figure 1.4 Soluble ErbB2 protein forms. Soluble ErbB2 protein exists in several forms due to a combination of alternate splicing and proteolytic processing. Full length ErbB2 protein (A) can be proteolytically processed to yield a 105 – 110 kDa protein (B). Alternative splicing of the ErbB2 gene can produce a 100 kDa (C) or a 68 kDa (D) soluble protein.

The soluble form of ErbB2 (sErbB2) has been shown to be released from breast cancer cells (Liu et al. 2006), and is recognised as a promising biomarker in profiling the progression of cancer, in particular, metastatic breast cancer where it is elevated in 30-70% of cases and associated with progressive metastatic disease and poor response to chemotherapy (Carney et al. 2003). Several studies have shown correlation between the presence of ErbB2-overexpressing tumours and elevated levels of sErbB2 in patient blood fractions (Fornier et al. 2005, Kong et al. 2012, Ludovini et al. 2008) and an sErbB2 protein level ≥ 15 ng/ml or a change of 20% in levels between two successive blood draws is significant to indicate further clinical investigation into ErbB2 positive cancer (Carney et al. 2013). Measurement of sErbB2 may provide a more informative diagnostic tool, both in terms of overall ErbB2 load and monitoring tumour response to therapy. ErbB2 is not a unique hallmark of a cancerous cell phenotype, and basal expression of ErbB2 is sometimes a feature of normal tissues (Negro et al. 2006). For this reason, it is unlikely that sErbB2 can be used as a sole and specific diagnostic marker of the presence of cancer in patients. However, an in-depth review of 22

studies has concluded that in the majority (85%) of individuals surveyed, sErbB2 levels correlated with disease recurrence, metastasis or shortened survival time (Ross et al. 2003). Taking this factor into consideration, the value of sErbB2 as a biomarker lies in assessing the metastatic state of ErbB2 cancers and seems to allow tracking of the response to anti-cancer treatments.

For example, decreased extracellular domain ErbB2 protein during Trastuzumab treatment has been suggested as a good predictive biomarker in terms of the positive response to cancer treatment, both in terms of time to disease progression and overall survival (Ali et al. 2008). However, a more in depth understanding of the mechanisms of synthesis and release of sErbB2 from ErbB2-positive tumours will further clarify the utility of sErbB2 in tracking response to cancer therapies (Lennon et al. 2009).

A test for sErbB2 is important since assessing sErbB2 in blood has the potential to give a global assessment of cancer burden within the body (rather than relying on a single biopsy sight), and furthermore it can serve as the first early indication of the presence of ErbB2-positive primary cancers, and in particular, more advanced metastatic cancer. Additionally, testing sErbB2 in blood is relatively non-invasive (as opposed to biopsy) as such this facilitates regular monitoring of sErbB2 levels in the body to treatment response. The main limitation with the current sErbB2 tests is that they are monoparametric, in that they are capable of measuring a single protein at a time (i.e., sErbB2). Since the real value of biomarker analysis in personalised medicine lies in the context of measuring multiple markers at one time, thus allowing comparative analysis within one individual over time, the current kits fall short on being able to do this.

Other members of the ErbB family of proteins also have soluble forms. ErbB1 (also known as EGFR) has a soluble form protein which is found circulating in blood and recent studies suggest that sEGFR may predict responsiveness to EGFR-targeted therapies (Wilken et al. 2013). However, for the moment, sErbB2 is the more clinically validated than other members of the ErbB family.

1.1.6 Nanotechnological answers to current diagnostic limitations

Current *in vitro* and *in vivo* clinical diagnostic practises have a number of inherent limitations which hamper development towards improved cancer treatment strategies.

At an *in vitro* diagnostic level, there is a routine reliance on a subjective, non-quantitative tumour grading system, namely immunohistochemistry (IHC), where parameters such as fixation may limit specificity of antigen detection (Penault-Llorca et al. 1994), resulting in both false-positive and false-negative results. These may have a direct impact on the diagnostic observation and patient treatment. Furthermore, biopsy studies suggest that there is discordant ErbB2 expression between primary and metastatic sites within the same patient (Lanitis et al. 2012), and highlights the shortfall in accurate cancer diagnosis through reliance on assessment of the primary lesion alone.

In terms of blood fraction analysis, sErbB2 correlates well with disease behaviour and shows potential to measure susceptibility to cancer recurrence and response to treatment (Ross et al. 2003). However, assessment of sErbB2 levels alone may not be sufficient to accurately predict cancer status. Due to the heterogeneous nature of cancer progression, it is recognised that the future of effective cancer profiling lies in the simultaneous grading of multiple tumour markers (Hirsch et al. 2009). Additionally, the importance of assessing circulating tumour cells (CTCs) as a prognostic indicator of progression free survival and response to targeted therapy has been highlighted by recent evidence (Liu et al. 2013). A number of commercially available kits are available for assessing ErbB2 in CTCs. However, the level of inter-kit agreement merits further investigation (Fehm et al. 2010).

In addition to ErbB2 quantification, there is a necessity for new techniques which measure the activity (e.g. degree of receptor dimerisation) of ErbB2 and other markers such as EGFR in tumour cells. This information can be gained by using techniques such as Förster resonance energy transfer (FRET) (Waterhouse et al. 2011) and may inform the clinician on the efficiency of targeted treatments by assessing the receptor activity (Gaborit et al. 2011).

In vivo imaging techniques have been used for many years to visualise tumour tissue. However due to the non-specific distribution of contrast agent such as in X-ray computed tomography (XRCT), and short renal clearance times, the patient is often exposed to high contrast agent dose during the imaging procedure. Passive *in vivo* contrast agents (e.g. Indocyanine Green) naturally accumulate in vasculature and tumour tissue due to the enhanced permeability and retention (EPR) effect (Napp et al. 2011). The EPR effect has been exploited in order to visualise tumours *in vivo*. However, EPR alone cannot provide the

information regarding the molecular profile of the tumour tissue. Therefore, there is a demand for cancer-targeted *in vivo* imaging strategies which could reduce the overall burden on the patient whilst increasing cancer detectability and informing the clinician on the molecular phenotype of the tumour.

Although current established molecular profiling techniques have gone a long way to advancing the standard of medical treatment, there is a need to move towards more quantitative, objective cancer diagnostic systems in tissue biopsy and blood fractions analysis. In short, there is a need to improve the reliability and accuracy of disease diagnosis and to guide the selection of effective treatment for patients.

Over the past decade, nanotechnology-based diagnostic approaches have emerged as promising candidates for the improvement of clinical diagnostic techniques. The development of a number of families of nanoparticles (NPs), development of chemical linkers and antibody engineering have the potential to greatly enhance ErbB2 assessment to the point of single molecule detection. In addition, the development of nanowire (NW) structures for multiplexed “lab-on-a-wire” bio-analytical devices (Patolsky et al. 2006, Zhang & Ning 2012) has shown advantages in high throughput assays when compared to conventional and NP-based probe systems (Prina-Mello et al. 2010) leading to the possibility of improved analyte detection sensitivity in biological assays.

The use of nanomaterials (NMs) (i.e. NPs and NWs) as biomarker sensors could overcome such shortfalls and limitations. In particular, NM-assays have two major advantages over conventional assays (Bhaskar et al. 2010): (1) they enable higher imaging sensitivity, (2) they can merge multiple modalities on one probe (for example, NMs that can efficiently target cancer cells allow combining biomarkers detection with *in vivo* cancer imaging) (Kumar 2007, Kumar 2006).

To date, a number of nanotechnological approaches have been translated to and approved for medical applications, and most of these are liposome-based and protein-polymer-based drug treatments (Etheridge et al. 2013, Schütz et al. 2013). In spite of the success in translating nanotechnology-based treatments to the marketplace (recent examples include Marquibo® & Bepanthen®), there has been comparatively little translation of nano-research to everyday clinical diagnostic practise and this stimulates the quest for further nanotechnological breakthroughs. Given the clinical importance of ErbB2, this literature review focuses on the NM-based technologies which aim to improve ErbB2 detection and quantification for diagnostic purposes.

The sErbB2 protein is used as a prototype model for the development of the nanowire platform, for a number of reasons. Firstly, ErbB2 is well recognised as a cancer biomarker, and sErbB2 is seen as a promising biomarker which could allow the non-invasive detection of ErbB2-positive cancers and may allow the ability to track disease response to therapy. Secondly, there are several EU-certified commercially available sErbB2 kits (although none of them are nanotechnology-based), which can be used as a comparative benchmark for the development of a nanowire-based assay. In essence, sErbB2 is being used as a test bed for the development of the nanowire platform. Once the principles of the nanowire assay have been established, the methodologies and experience could be applied to adapt the nanowire platform to the detection of other biomarkers (for example, EGFR and PSA as is demonstrated in Chapter 4).

1.2 Clinical methodologies for the detection and quantification of ErbB2

Clinically, the molecular presence of ErbB2 in tumour tissue is identified by *in situ* hybridisation (ISH), which characterises ErbB2 gene copy number, and IHC, which measures ErbB2 protein content. Enzyme-linked Immuno-Sorbent Assay (ELISA) is one of the most common methods to quantify sErbB2 levels in patient blood fractions (Carney et al. 2004).

1.2.1 Genetic detection and quantification of ErbB2

All DNA assessment techniques measure the predisposition potential toward a given proteomic phenotype and thus can act as an early detection system for cancer. Polyploidy is a common feature of cancer cells (Storchova & Pellman 2004, Zhang et al. 2013), and so measurement of the gene copy number can indicate the presence of pathological cells. In clinical practise, genetic assessment of ErbB2 is generally used in conjunction with IHC results in order to confirm ErbB2 status in biopsy tissue.

In ISH, the nucleic acid probe is hybridised to its complementary nucleic acid strand in the tissue section. Hybridisation allows the visualisation and enumeration of specific gene loci (Fig 3) via a direct or indirect fluorescent (i.e. Fluorescent *in situ* hybridisation; FISH) or chromogenic (i.e. Chromogenic *in situ* hybridisation; CISH) tag. mRNA ISH (see section 1.2.1.3) allows examination of the ErbB2 mRNA transcripts within the tissue section. Genomic hybridisation arrays and real-time polymerase chain reaction- (RT-PCR) based assays can measure a vast amount of genetic loci or gene transcripts at one time

1.2.1.1 Fluorescent *in-situ* hybridisation (FISH)

Commercially available FISH assays for ErbB2 gene assessment (summarised in Table 1.2) employ direct labelling of the DNA with a complementary DNA probe (which is directly conjugated to a fluorophore, such as Spectrum Orange™ in the PathVysion™ kit and TexasRed® in the Dako IQFISH™ kit) (Fig. 3). FISH assays are usually restricted to formalin-fixed paraffin-embedded tissue sections and probe hybridisation is carried out for 8-10 hours. Even though they are considered one of the gold standard techniques for the clinical profiling of tumours, successful use of FISH kits for ErbB2 quantification relies heavily on expert execution of complex laboratory protocols. On this topic, Cayre et al. (2007) have published an extensive comparative report on the different experimental outcomes obtained from a variety of commercially available ErbB2 FISH kits tested on the same samples. They concluded that the tested are very well in accordance with one another. However, this agreement was reliant on the relative adjustment of signal threshold levels. The technique appears therefore to be susceptible to user error. Finally, the resolution (ability to distinguish between two points on a chromosome length) of *in situ* hybridisation assays can also be affected by the cell cycle phase (O'Connor 2008).

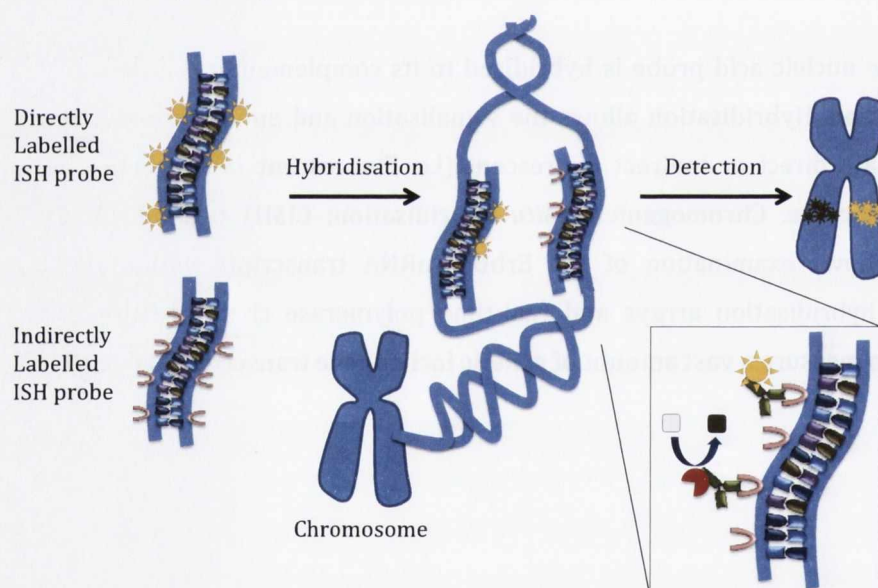


Figure 1.5 Principle of *In-Situ* Hybridisation. This figure represents the principles of ISH using either a directly- or indirectly- labelled method. In directly-labelled ISH, the DNA probe is directly conjugated to a fluorophore (yellow), as occurs in FISH. In indirectly-labelled ISH, the DNA probe is conjugated to a hapten (pink). During the denaturation step of the ISH protocol, the DNA probe hybridises to the gene of interest. Directly-labelled probes can then be visualised. Hapten-conjugated probes are detected either with a chromogen-linked antibody (red)(CISH), or with a fluorescently-labelled antibody.

1.2.1.2 Chromogenic *in-situ* hybridisation (CISH)

CISH allows the genetic assessment of tissue biopsies at lower magnifications compared to FISH. Thus, CISH can be used to measure gene ploidy within the greater context of the surrounding tissue. Although the resolution of CISH may not reach that of FISH, it is less costly and does not require specialised equipment, and so may be more suited to certain clinical situations.

CISH relies on an indirect labelling method, whereby the DNA probe is conjugated to a hapten (e.g. INFORM or Spot-light kits, Table 1.2) against which an antibody is raised. Such antibodies are tagged with a chromogenic marker rather than a fluorophore as occurs in FISH. Interestingly, products supplied by Dako Denmark A/S use identical DNA probes in their FISH and CISH kits, with the latter simply adding antibodies against the FISH fluorophores (TexasRed® and Fluorescein). The chromogen is then converted to a visible product, usually by an enzymatic reaction (e.g. by addition of horseradish peroxidase (HRP) or alkaline phosphatase (AP)). CISH brings with it a few advantages over FISH assays. Firstly, indirect labelling with anti-hapten antibodies permits optical detection of ErbB2 genes at lower magnifications. Secondly, CISH stained samples can be archived without incurring sample or staining degradation (Cayre et al. 2007). This advantage might be counteracted by incorporating stable nanotechnology-based fluorophores into FISH techniques.

1.2.1.3 mRNA *in-situ* hybridisation (mRNA ISH)

mRNA ISH quantifies the ErbB2 gene expression levels. This technique has some unique advantages over FISH and CISH assays. Firstly, mRNA ISH detects ErbB2 gene transcripts and thus is more representative of the ErbB2 transcriptome levels in tumour cells. A recent study has shown, however, that there is a close correlation between mRNA ISH and FISH when determining ErbB2 levels in primary breast tumours (Alba et al. 2012). Secondly, the reduced processing time (4 h) of samples by mRNA ISH makes this technique advantageous when compared to FISH and CISH approaches. Affimetrix's QuantiGene ViewRNA kit (Table 1.2) can detect up to four different RNA transcripts at a time. In this instance, branched DNA amplification technology (Tsongalis 2006) attached to the mRNA probe is used to amplify the captured target RNA (rather than relying on an *in vitro* reverse transcriptase amplification of mRNA) (Horn et al. 1997), and visualisation of the amplified RNA is tailored for brightfield, fluorescent or luminescent detection. As an alternative to mRNA ISH, Adnagen (Germany) (Table 1.2) have developed a RT-PCR-based diagnostic which measures ErbB2 mRNA content as one of a panel of mRNA markers in CTCs. This is achieved through immunomagnetic concentration of CTCs from a blood sample followed by mRNA analysis. The technique assesses ErbB2 positivity through measurement of the final concentration of amplified cDNA.

1.2.1.4 Genomic hybridisation arrays

Hybridisation arrays consist of immobilised DNA probes on a solid matrix microarray, which can be used to measure gene copy number variations (the signal intensity correlates with hybridisation intensity) and to compare gene expression profiles among normal and tumourigenic DNA profiles (Cavallaro et al. 2012). In contrast with the FISH approach, DNA (or cDNA generated from mRNA) for hybridisation arrays can be isolated from both fresh biopsy tissue and formaldehyde-fixed paraffin-embedded tissue.

Several microarrays are commercially available for assessing ErbB2 gene status. The FDA-approved MammaPrint (Agendia, Amsterdam) microarray assesses 70 genes (one of which is ErbB2 gene) associated with the development of distant metastases 5 years after surgery (Slodkowska & Ross 2009). The Oncotype DX (Genomic Health, Inc., CA) microarray also measures ErbB2 levels as one of sixteen tumour markers in comparison to five reference genes (Cronin et al. 2007). One of the main advantages of microarrays is that they allow the simultaneous analysis of a number of genes which may better reflect the heterogenic nature of tumour development.

1.2.1.5 Appraisal of ErbB2 gene detection techniques.

Table 1.2 details clinical diagnostic assays for the detection and quantification of tissue ErbB2 at a genetic and post-transcriptional level. From a technical standpoint, in ISH the efficiency of probe hybridisation into native DNA is skewed towards detection in cells in interphase of the cell cycle (O'Connor 2008) and often considerable specialised equipment is required for some of the genetic assessment platforms. Microarray-based assays allow simultaneous quantification of numerous genes. However, the technique may also be more sensitive to contaminating DNA. ErbB2 mRNA quantification techniques (such as that developed by Adnagen) which rely on RT-PCR in order to generate a detectable signal are ultimately limited by the quality and preparation of the sample mRNA.

One of the major limitations of the genetic detection/quantification methods for ErbB2 lies in the design of the DNA probe used. There is some preliminary evidence reporting the inaccuracy of ErbB2 FISH kits as determined by comparative gene hybridisation array analysis (McArthur et al. 2009). Despite the fact that the ErbB2 gene comprises a 40kb genomic region, most of the commercial ErbB2 probes currently commercialised for FISH/CISH assays consist of 190-218kb length DNA probes (Vysis PathVysion: 190kb, Ventana INFORM: 200kb and Dako IQFISH: 218kb, see Table 1.2) which may increase the chance of non-specific binding events (Allison 2010). As one solution, Invitrogen has recently

developed a proprietary 'subtractive hybridisation' technology (included in the SPOT-Light CISH kit), which removes repetitive sequences from its probes and generates higher specificity for the targeted ErbB2 gene. The incorporation of brighter fluorescent probes (based, for example, on quantum dot technology, as discussed in section 3) might further allow improved gene resolution and gene quantification assays.

Table 1.3 Diagnostic assays for ErbB2 gene and mRNA. The table details those commercially available assays used for clinical profiling of ErbB2 at a gene and mRNA level. The table was compiled based on on-site assay use and from a web search for ErbB2 diagnostic assays.

<u>ErbB2 form detected</u>	<u>Detection method†</u>	<u>Manufacturer (Country)</u>	<u>Assay Commercial Name</u>	<u>Declared Sensitivity</u>	<u>Clinical sample*</u>
2.1 <u>Gene/mRNA encoding for ErbB2</u>	<u>FISH</u>	Abbott Molecular, Inc. (USA)	PathVysion™ HER2 DNA Probe Kit	Ratio to reference gene included in kit	FFPE
		Dako Denmark A/S (Denmark)	HER2 IQFISH pharmDx™		
	<u>CISH</u>	Ventana Medical Systems, Inc. (USA)	INFORM™ HER2	Not stated	
		Invitrogen Corporation Ltd (USA)	SPOT-Light® HER2 CISH Kit	Single gene copy	
		Dako Denmark A/S (Denmark)	HER2 CISH pharmDx™	Ratio to reference gene included in kit	
		Affimetrix, Inc. (USA)	QuantiGene® ViewRNA	Single RNA molecule detection	
	<u>Microarray</u>	Genomic Health, Inc. (USA)	Oncotype DX™	Ratio to 5 reference genes included in kit	
		Agendia (Netherlands)	MammaPrint®	50% tumour cell content	Fresh tissue
<u>mRNA from circulating tumour cells</u>	<u>Immunomagnetic separation and RT-PCR</u>	Adnagen, Gmbh. (Germany)	AdnaTest BreastCancerSelect/ BreastCancerSelect	30ng/µl amplified cDNA	S
† FISH: Fluorescent <i>in-situ</i> hybridisation; CISH: Chromogenic <i>in-situ</i> hybridisation; RNA ISH: RNA <i>in-situ</i> hybridisation; RT-PCR: Real Time – Polymerase Chain Reaction. * FPE: Formalin-fixed paraffin-embedded; S: Serum.					

It is interesting to note the lack of standardised approach to assessment of ErbB2 DNA/RNA between the different kits, in terms of declared sensitivity (see Declared Sensitivity column in Table 1.2) of each assay.

1.2.2 Detection and Quantification of ErbB2 protein

The following sections address two of the conventional diagnostic approaches to quantify ErbB2 protein in tumour tissues and patient fluids. IHC represents the most commonly used clinical diagnostic method for semi-quantitative assessment of protein levels within the morphological context of tumour tissue biopsies (Table 1.3). IHC is a well-established and validated technique too, and no specialised equipment is needed. ELISAs are the main method for measuring soluble ErbB2 in blood fractions (Table 1.4). Since numerous studies correlate elevated blood sErbB2 levels with the presence of ErbB2-positive breast cancer tumours (Carney et al. 2004), sErbB2 ELISA is seen as a possible early detection system for ErbB2-positive cancers.

1.2.2.1 Immuno-histochemical detection of ErbB2 protein

IHC relies on using one specific antibody against the ErbB2 protein (Figure 1.6). Amplification of the signal is achieved by using enzyme linked- (e.g., HRP, AP or AP-ant-A₂) secondary antibodies guided against the detection antibody. The enzyme-linked antibody converts a substrate to a visible colour. Although good sensitivity can be achieved using this detection method, IHC uses only one ErbB2-specific antibody and non-specific binding phenomena can occur.

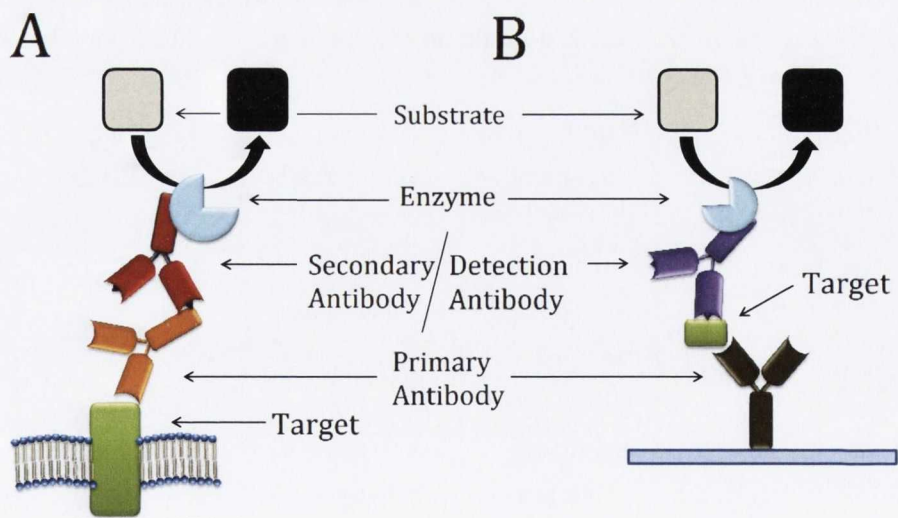


Figure 1.6 Principle of (A) IHC and (B) sandwich ELISA. (A) In IHC, detection of the antigen (green) is accomplished by an antigen-specific primary antibody (orange), followed by detection and signal amplification via an enzyme (light blue) -linked secondary antibody (red).

in the presence of a substrate. (B) In ELISA, both antibodies are directed against the antigen (green). The first antibody (grey) is immobilised on an assay well. Following capture of the antigen, the enzyme-linked detection antibody (purple), generates the chromogenic signal.

Various IHC kits for ErbB2 measurement are available, such as that produced by Dako (Denmark) using Trastuzumab as the detection antibody. Whilst Trastuzumab targets the extracellular domain of ErbB2, Biogenex and Ventana Medical Systems have developed their own ErbB2 IHC kits with antibodies (mouse monoclonal antibody CB11 and rabbit monoclonal antibody 4B5, respectively) guided against the intracellular portion of the ErbB2 protein. Trastuzumab has a simpler access route to ErbB2 (effective CB11 and 4B5 staining relies on successful penetration of permeabilised cells) and so is less likely to suffer from high background, non-specific staining. However, since there are sometimes a number of active, carboxy-terminal ErbB2 variants (which lack the extracellular domain) present in cells (Arribas et al. 2011), there is a chance that IHC assessment via Trastuzumab may underestimate the true amount of active ErbB2 in the cell. A threshold of >10% tissue staining is regarded as the minimum threshold for defining ErbB2 positivity by ErbB2 protein IHC kits (Table 1.3). Therefore, although IHC for ErbB2 is well established and widely used, the limited resolution of this technique makes it unsuitable for weakly expressed targets or for single-molecule detection. IHC gives the clinician a “reasonable” assessment of ErbB2 tissue load. However, there are a number of limitations related to this technique. IHC is semi-quantitative, subjective, not sensitive at low ErbB2 expression ranges (0 to 1+) and reliant on activity of staining reagents (e.g., peroxidase activity)(True & Gao 2007).

Table 1.4 Tissue diagnostic assays for the detection and quantification of human ErbB2 protein. The table details those commercially available assays used for clinical profiling of ErbB2 at a protein level. The table was compiled based on on-site assay use and from a web search for ErbB2 diagnostic assays.

<u>Tissue ErbB2 form detected</u>	<u>Detection method[†]</u>	<u>Manufacturer (Country)</u>	<u>Assay Commercial Name</u>	<u>Declared Sensitivity</u>	<u>Clinical sample tested*</u>
<u>ErbB2 protein</u>	<u>IHC</u>	Biogenex (USA)	InSite™ HER2/neu CB11	>10% Tissue Staining	FFPE
		Dako Denmark (Denmark)	Hercep Test™		
		Ventana Medical Systems, Inc. (USA)	Pathway® anti HER2/neu (4B5)		
	<u>Proximity-</u>	Monogram	HERmark®	Not stated	

	<u>based</u>	Biosciences (USA)	Breast Cancer Assay		
† IHC: Immuno-histochemistry.					
* FFPE: Formalin-fixed paraffin-embedded.					

1.2.2.2 Enzyme-linked Immuno-Sorbent assays (ELISAs)

As illustrated in table 1.4, a number of sErbB2 ELISA kits are available for quantification of sErbB2. ELISAs use two specific, target-directed antibodies (capture and detection antibodies), followed by an enzyme-driven amplification step (e.g., *via* HRP- or AP-linked secondary antibodies) (Figure 1.4B) with quantification of antigen achieved through comparison to a standard range. This approach increases the probability of specific detection of sErbB2. Thus, ELISA is a quantitative rather than a semi-quantitative assay. Clinically, the FDA-approved cut-off concentration above 15ng/ml for sErbB2 is considered indicative of the presence of ErbB2-overexpressing cancers (Carney et al. 2004).

Table 1.5 ELISAs for the detection and quantification of human ErbB2 in biological liquid fractions. The table details those commercially available ELISAs used for clinical profiling of soluble ErbB2 at a protein level. The table was compiled based on on-site assay use and from a web search for ErbB2 diagnostic assays.

<u>Soluble</u>	<u>ErbB2</u>	<u>Manufacturer</u>	<u>Assay Commercial Name</u>	<u>Declared</u>	<u>Clinical</u>
<u>Detection method†</u>	<u>(Country)</u>			<u>Sensitivity</u>	<u>sample tested*</u>
<u>ELISA</u>	WILEX, Inc. (USA)	Her2/neu ELISA	1.5 ng/ml	S	
	Abcam, plc (UK)	ErbB2 Human ELISA kit	8 pg/ml	U, S, P	
	Ray Biotech, Inc. (USA)	RayBio® Human ErbB2 ELISA kit	8 pg/ml	U, S, P	
	eBioscience, Inc. (USA)	Human sHER-2 Platinum ELISA	0.06 ng/ml	S, P	

† ELISA: Enzyme-linked Immuno-sorbent assay

* S: Serum; U: Urine; P: Plasma

ELISAs have a number of advantages over IHC. ELISAs are quantitative, easy to use, have relatively short assay time and have very sensitive detection limits. Furthermore, ELISA is able to assess the pooled sErbB2 circulating in blood, which could give a better indication of the overall cancer status of the patient. However, there is some evidence of sErbB2 false positive in patients without cancer, with elevated levels of sErbB2 being reported in cases of

liver cirrhosis (Molina et al. 1997). However, this does not negate the utility of assessing sErbB2 as a cancer detection strategy. The drawbacks associated with ELISAs include high cost of the assay, usually only mono-parametric detection (Table 1.4) and the absence of histological context of protein quantification. ELISA is also unsuitable for quantification of CTCs.

1.3 Enhancing ErbB2 detection using nanotechnology-based tools

Despite the advances in the development of genetic and protein assessment technologies, there is now a requirement to bring clinical diagnostics to new levels of molecular specificity and sensitivity. From a clinical standpoint, NMs provide the vehicle with which to improve routine clinical diagnostic techniques with respect to signal detection fidelity, single cell and analyte detection sensitivity and multiplexing of biomarker analysis. In the case of ErbB2 detection, there is a clinical need for gene probes with improved specificity (see limitations in section 1.2.1.5) and protein probes with improved signal-to-noise ratio for the accurate determination of circulating biomarkers (Lam et al. 2012).

1.3.1 Nanomaterials for molecular imaging and detection purposes

In recent years, exploitation of the inherent properties of NMs (such as quantifiable optical, magnetic and plasmonic properties, altered catalytic properties, high surface-area to volume ratio, and the possibility of surface modification) has facilitated higher sensitivity and specificity in biomarker detection. As a consequence, there is a wealth of NM-based technologies under development for biomarker detection utilising quantum dots (QDs), superparamagnetic iron oxide NPs (SPIONs), noble metal NPs such as gold NPs (Au NPs) and silver NPs (Ag NPs), carbon nanotubes (CNTs) and silica nanowires (Si NWs) (Chi et al. 2012, Ray et al. 2011).

The use of chemical linkers such as polyethylene glycol (PEG), (3-Aminopropyl) triethoxysilane (APTES) and dextran has enabled functionalisation of NPs with bioactive molecules such as antibodies, ligands, biocompatibility agents and drug candidates (Bhowmick et al. 2012, Costas & Olga 2013, Martin et al. 2009) and has opened up the possibility of exploiting NPs for biological detection. This development has allowed NPs to be aimed against specific targets for visualising molecular interactions (such as immune function) and investigating therapeutic pathways (Walling et al. 2009). For example, surface modification of NPs with PEG has permitted the conjugation of engineered ultra-small single domain antibodies to super-bright stable fluorescent NPs (QDs, discussed in section 3.2.1.2) (Figure 1.5) (Sukhanova et al. 2011). This synthesis allows twice as many antibody binding

sites to be incorporated into the same space as a monoclonal antibody and is currently being developed for targeting ErbB2 in cell populations using flow cytometry (Sukhanova et al. 2012). In the *in vivo* setting, functionalised superparamagnetic NPs have been successfully investigated for use as tumour-targeted nuclear magnetic resonance imaging (NMRI) contrast agents (Hathaway et al. 2011). Furthermore, it is hoped that the functionalisation of NPs with cancer-targeting moieties will reduce the administered dose used for *in vivo* tumour imaging. The following sections illustrate the NP-based technologies being developed for the advancement of *in vitro* and *in vivo* ErbB2 cancer diagnostics and imaging.

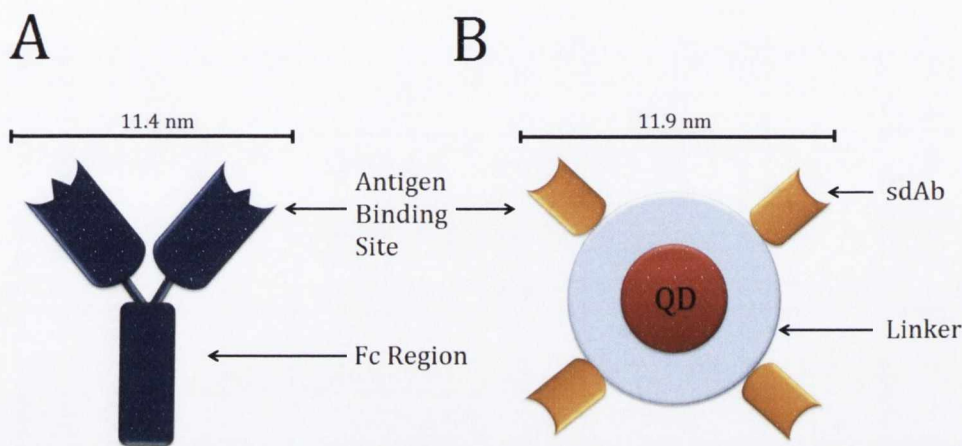


Figure 1.7 Size comparison between (A) conventional immunoglobulin and (B) immune-functionalised quantum dot. Abbreviations: sdAb: single-domain antibody fragment. Adapted from Sukhanova et al 2011.

1.3.2 Nanoparticle applications in *in vitro* ErbB2 assays

Some NP applications for *in vitro* detection of ErbB2 are listed in Table 1.5. An overview of the technical details associated with each NP format is given, and an attempt is made to evaluate the potential for these technologies to transition effectively to clinical practise.

Although some attempts have been made to apply NPs to *in vitro* genetic assessment of ErbB2 (Ioannou et al. 2009, Xiao & Barker 2004), mixed successes have been observed. The vast majority of NP technologies applied in ErbB2 profiling are based on detecting the protein form rather than the gene target, providing significant progress towards improving current diagnostics. Section 3.2 discusses the applications of nanotechnologies to ErbB2 *in vitro* diagnostics.

Table 1.6 Nanoparticle applications in *in vitro* detection and imaging of ErbB2 in tissue. The technical details section gives information on the nanoparticle format, its demonstrated application, as well as the declared sensitivity of each system if mentioned. Within each ErbB2 form detected (left-most column), the approaches are ranked in terms of their potential to affect clinical translatability. Clinical translatability was assessed primarily under ability to specifically detect ErbB2, comparison to conventional techniques, improved aspects/limitations, sample type examined and ease of use.

Technical Details					Clinical Translation Details			
Tissue ErbB2 form <u>detected</u>	Detection/ Imaging <u>method</u> [†]	Nanoparticle <u>Format</u> *	Sample <u>tested</u>	Sensitivity <u>declared</u> [±]	Improvement over existing <u>technology</u> ^o	Limitations ^{**}	Score ^{oo} 1-5	Reference
ErbB2 Gene	FISH	QD-labelled gene probes	Lymphocytes & SKBR-3 cell line	Low gene copy number detection	Improved fluorescent properties over organic dye (FITC).	Improved sensitivity over existing technique not demonstrated.	3	Xaio & Barker 2004
Total ErbB2 Protein	Fluorescence Microscopy	QD-IHC: QD-SA used to detect biotinylated antibody.	Fixed tissue sections and fresh biopsy arrays	Equal to IHC for ErbB2, improved for ER.	Multiplex of analyte detection with better ER quantification than IHC. Good concordance with IHC ErbB2 scores.	More expensive than IHC.	4	Chen et al 2010
	Fluorescence microscopy	QD-IgG or QD-SA	SKBR3 cell line and tumour tissue		Superior fluorescence properties vs organic dye. Multiplexed imaging.	Improved detection limit not demonstrated in tumour tissue.	4	Wu et al 2002
		Cyanine Itrybe-doped polystyrene NPs + IgG	Cell lines and tumour tissue		Superior NIR emission properties vs organic dyes. Xenograft tissue tested.	Comparison to conventional techniques not shown.	4	Behnke et al 2013.
		QD-cys	Cell lines:		Multiplexed detection of ErbB2	Comparison to	4	Barat et al 2009

Cell surface ErbB2	Microscopy/ FACS	diabody conjugate	MCF7, SKOV-3, LNCaP/PSCA	Variable depending on cell type	and PSA. Specific orientation of miniaturised diabody.	conventional techniques not shown.		
	Fluorescence microscopy and magnetic separation	SPION-SiNP coupled to antibody	Cell lines SKBR-3 and NIH/3T3.		Multimodal separation and detection of ErbB2 positive cells.	Comparison to conventional techniques not shown. Low ease of use.	3	Mi et al 2011
	MRI	SPION coupled to antibody	Cell lines SKBR-3 and H520		Improved magnetic relaxation over existing commercial alternatives.	Comparison to conventional techniques not shown. Low ease of use.	3	Yang et al 2010
			Cell lines SKBR-3 and SKOV-3	Average 3 CTCs per sample	Improved CTC detection rate compared to existing commercial alternative. Measures four CTC markers.	Device optimisation still underway.	5	Ghazani et al 2012
	Two-photon scattering	Noble Metal nanoparticle coupled to antibody and RNA-aptamer	Cell lines SKBR-3 & MDA-MB-231	100cells/ml	Label-free detection. Very sensitive SERS-based detection of ErbB2-positive cells.	Not demonstrated in mixed cell populations.	3	Lu et al 2010
	Fluorescence microscopy	QD-doped, antibody-linked polyacrolein particles	Cell line SKOV-3	Cell type specific detection of ErbB2	Mini-antibody allows multipoint binding sites for cell surface ErbB2 binding	Platform not fully tested for ErbB2 compared to <i>Y. pestis</i> antigen.	2	Generalova et al 2011
	Fluorescence	Iron	Fresh Human	1 CTC per 7.5ml	Simplified CTC separation from	Commercialised product.	5	CellSearch®,

	microscopy/ Magnetic sorting	Nanoparticle	Blood	blood	complex biological sample.		Janssen Diagnostics, USA
	Spectroscopic measurement of plasmonic coupling	Noble Metal nanoparticle coupled to antibody	Cell SKBR-3	ErbB2 Receptor dimerisation	Assesses receptor dimerization. Possible new aspect to clinical assessment of cancers.	Comparison conventional techniques not shown. Low ease of use.	Crow et al 2011
Cell Lysate ErbB2	Electrochemi cal conductivity	Noble Metal nanoparticle coupled to antibody	Cell lysate from SKBR-3 and MCF7	ErbB2 protein 100ng/ml	Improved signal to background ratio compared to conventional Gold-electrode.	Lower detection limit compared to ELISA. Manufacture complexity vs ELISA.	Mucelli et al 2008
† FISH: Fluorescent <i>in-situ</i> hybridisation; FACS: Fluorescence-activated cell sorting; MRI: Magnetic Resonance imaging; κ QD: Quantum Dot; IHC: Immuno-histochemistry; IgG: Immunoglobulin; SA: Streptavidin; NP: Nanoparticle; SPION: Super paramagnetic Iron oxide nanoparticle; SiNp: Silica Nanoparticle. ± ER: Estrogen receptor. ° FITC: Fluorescein isothiocyanate; NIR: Near-infrared; PSA: Prostate-specific antigen; SERS: Surface-enhanced Raman spectroscopy. **ELISA: Enzyme-linked Immuno-sorbent assay.							

1.3.2.1 Nanoparticles in optical detection of ErbB2:

Fluorescent quantum dots

Quantum dots (QDs), described as nanocrystals composed of semiconductor materials, hold intrinsic properties which could vastly improve the quality, fidelity and durability of optical imaging for biomarker detection. QDs exhibit superior fluorescent quantum yield at NIR wavelengths compared to organic dyes (0.05–0.25 for organic dyes vs 0.2–0.7 for quantum dots), higher photostability at NIR wavelengths (an advantage in archiving of biological samples), broad absorption spectra, narrower, Gaussian emission spectra, tuneable emission wavelengths and longer fluorescence lifetime decay and their small dimensions allows them to penetrate tissue barriers (Resch-Genger et al. 2008). In terms of fluorescent properties, QDs far outperform the commonly used FISH kit organic fluorophores such as fluorescein (Larson et al. 2003) and Texas Red® (Smith et al. 2006) and have been applied in a FISH detection system (Xiao & Barker 2004).

Another reason for using QDs for diagnostic assays is that multiple QDs can be excited by a single non-coherent light source, such as an ultra-violet (UV) lamp (Figure 1.6). This reduces the need for specialised equipment and associated costs. As with organic dyes (Wang et al. 2010), QDs can have tuneable emission wavelengths (Smith et al. 2006), meaning they can be tailored to various detection and imaging applications. The synthesis of QDs of various emission spectra is largely a time dependent reaction (allowing the synthesis reaction to proceed results in larger QDs with a longer wavelength emission peak), and may be a simpler synthesis than creating a range of organic dyes with similar emission peaks. Therefore, tuning QDs emission peaks can be tuned to desirable wavelengths by modifying their size (Smith et al. 2006), allowing the development of simple-to-use, QD-based, multiplexed assays (Chen et al. 2010).

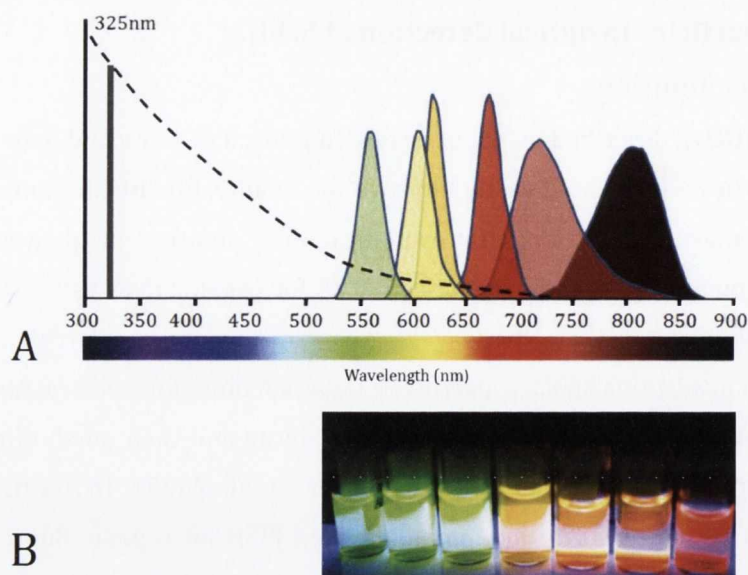


Figure 1.8 (A) Absorption (dotted line) and emission (coloured curves) spectra of QDs. In general, QDs strongly absorb short wavelength radiation. Emission spectrum peaks represent the wavelength at which maximum fluorescent intensity can be measured from an excited QD. The emission wavelength is relative to QD size, assuming QD composition is the same. Commercially available QDs excited by a single UV light source (e.g. 325nm) fluoresce in the visible and near-infrared spectrum (500-800 nm). (B) Image of QDs in solution under fluorescent excitation. Image courtesy of Dr A Rakovich (Imperial College London).

Optical detection of ErbB2 protein *in vitro* using quantum dots

QDs have been used for the detection of ErbB2 in IHC-based assays and are being developed toward the detection of rare CTCs in patient blood fractions (Mahmoud et al. 2010). QDs conjugated to streptavidin and incorporated into a conventional IHC protocol can act as a signal amplifier for biotinylated secondary antibodies (Howarth et al. 2005, Wu et al. 2003) (Figure 1.7). Wu et al. (2003) demonstrated multiplexed detection of ErbB2 and nuclear antigens using QD-IgG or QD-streptavidin, and showed the favourable photostability of QDs over organic dyes. In tumour tissue samples, ErbB2 protein has been detected in breast cancer core biopsy using QDs (ZnS/CdSe, 605nm emission peak), with improved results over traditional IHC in terms of quantification of ErbB2 (Chen et al. 2009, Chen et al. 2010). In this case, the improved visual contrast in QD-stained samples compared to traditional ErbB2 IHC allowed software-based statistical analysis of the tissue sections in terms of fluorescence intensity and distribution area. Using two different emission spectrum QDs (605nm and 545nm) this approach was successfully used to simultaneously image ErbB2 and ER in breast cancer sections (Chen et al. 2010).

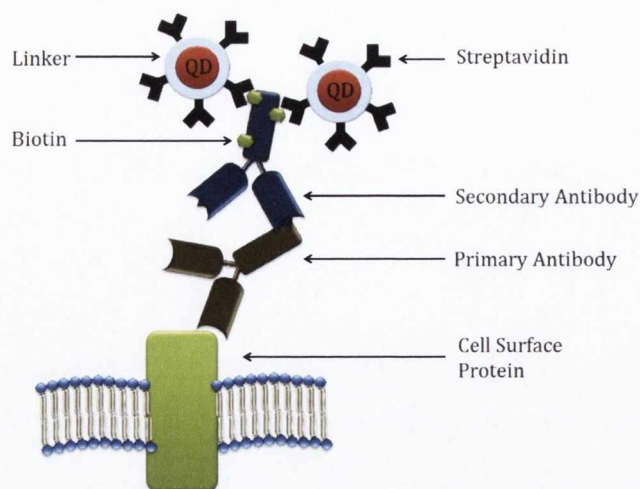


Figure 1.9 Schematic of Streptavidin-coupled QD for detection of proteins through binding to biotinylated secondary antibody (Adapted from Howarth et al. (2005)).

QDs have been impregnated into submicron polyacroleine particles, followed by antibody functionalisation, to create an ErbB2 probe for *in vitro* cell imaging (Generalova et al. 2011). Although QDs have also been applied to the assessment of ErbB2 gene copy number (Xiao & Barker 2004), and improved fluorescent properties over an organic dye were demonstrated, a more recent publication (Ioannou et al. 2009) has shown that in the case of FISH, the reproducibility of ErbB2 gene detection was more difficult with QD-based assays.

QD-based IHC performs well at detecting low level expression of ErbB2 in tissue (Chen et al. 2009). By utilising fluorescent-linked probes rather than enzymatically-linked probes, QDs help to move IHC from a semi-quantitative, subjective diagnostic technique toward a quantitative, objective one. This NP-based application has the potential to improve current clinical diagnostics through standardisation of tissue scoring systems.

Multiplexed fluorescence-activated cell sorting (FACS) using quantum dots

FACS allows the quantitative, multiplexed examination of molecular markers within a cell population by simultaneously processing multiple fluorophore signals, each fluorophore being linked to a specific marker. Due to their inherent photostability and tuneable fluorescent emission wavelength, the use of quantum dots rather than organic dyes is set to improve the performance of FACS as a diagnostic technique. FACS has been used to detect surface ErbB2 expression in cancer cell lines in suspension using fluorescent QDs directly conjugated to ErbB2-specific antibody fragments (cys-diabodies) (Barat et al. 2009) and the selective detection of ErbB2-positive cells (MCF7, SKOV-3) vs. prostate specific antigen-expressing cell lines (LnCap, PSCA) using QDs has been demonstrated. Although this work

was carried out using cell lines in solution, it may be possible to apply QDs to CTC detection in patient blood fractions.

To date, QD-based techniques have shown more promise for improving the quality of ErbB2 protein assays rather than ErbB2 gene assays *in vitro*. The combination of their stable optical properties, functionalisation potential and nanometer size has allowed QDs to be adapted to different diagnostic platforms such as IHC and FACS (Barat et al. 2009, Barteneva & Vorobjev 2010, Chen et al. 2009). This adaptability has significant future implications in terms of increasing the reliability of comparison between different diagnostic techniques.

1.3.2.2 Detection of ErbB2 using metal nanoparticles

Noble Metal nanoparticles

Gold (Au) and silver (Ag) NPs possess a number of properties (e.g. localised surface plasmon resonance capable of enhancing Raman scattering from biomolecules, electroconductivity, photoelectric potential, biocompatibility, lack of photobleaching) which can be exploited in clinical diagnostic techniques for the detection of ErbB2 (Maiti et al. 2010, Samanta et al. 2011). Table 1.6 below lists some of the main physical properties of Au and Ag NPs alongside their application in the detection and imaging of biomarkers.

Table 1.7 Main physical properties of Gold and Silver Nanoparticles. The left column shows the property with the corresponding application listed in the right column.

<u>Gold and Silver Nanoparticle Features</u>	<u>Bioimaging Application</u>
Localised Surface Plasmonic Resonance	Surface Plasmon Resonance Imaging
	Surface Enhanced Raman Spectroscopy
Two Photon Scattering	Two Photon Emission Microscopy
X-Ray Radiopacity	X-Ray Computer Tomography
Electroconductive	Electrochemical Detection

Localized Surface Plasmon Resonance (LSPR)

Noble metal NPs (such as Au NPs and Ag NPs) exhibit localized surface plasmon resonance (LSPR), which can be defined as enhanced optical absorption and scattering at a specific peak resonant wavelength. This property is tuneable by altering composition, size and shape of the NPs, as well as the surrounding dielectric medium (Kelly et al. 2003). Seekell *et al.* (2011) demonstrated the multiplexed detection of ErbB2 along with two other cancer biomarkers in various cancer cell populations using antibody-functionalised Au NPs and Ag NPs in flow cytometry. Additionally, LSPR has been used to study receptor dimerisation of ErbB2 by observing plasmonic coupling between adjacent NPs (Crow et al. 2011), adding an additional interrogative power to future assay development.

Surface-Enhanced Raman Spectroscopy (SERS)

Raman spectroscopy is a non-destructive, non-invasive optical method which is based on the inelastic scattering of light (Lyng et al. 2007). Metal NPs such as Au NPs and Ag NPs, possess localised surface plasmon resonance which enhances Raman scattering from nearby molecules. This property has led to their use in surface-enhanced Raman spectroscopy (SERS). Since enhancement of Raman scattering from biomolecules is dramatically increased in the presence of metal NPs, often by several orders of magnitude, it makes metal NPs ideal

for use in detection in a complex system. For example, binding of an analyte to functionalised metal NPs will cause an amplification of the Raman spectrum of that analyte, indicating that analyte binding has occurred.

The detection of ErbB2-positive cancer cells *in vitro* using SERS has been achieved by coupling antibodies to the surface of Au NPs (Maiti et al. 2010). In this study, Raman scattering was measured through the skin of nude mice following subcutaneous injection of Au NPs. In a follow-up study, the same group demonstrated SERS detection of cancer xenografts in mice, using functionalised Au NPs (Samanta et al. 2011). Similarly, Ag NPs have been used to differentiate between ErbB2-positive and ErbB2-negative cell lines using SERS. Improvements in the design and functionalization of NM-based SERS detectors will ultimately facilitate the widespread use of SERS for diagnostic purposes.

These studies demonstrate the potential application of targeted noble metal NPs in ErbB2 assays. Since this technique is not ideal for *in vivo* cancer imaging at depths greater than a few millimetres, SERS is likely to become a replacement or companion to *in vitro* cancer diagnostic kits (Porter et al. 2008).

Two photon scattering

Another result of the surface plasmon resonance displayed by metal NPs is the enhanced scattering of incident light (termed two photon scattering or hyper-Rayleigh scattering) at the second harmonic wavelength. Functionalised gold NPs have been used to discriminate between ErbB2-positive (SKBR-3) and -negative (MDA-MB-231) cell lines in a PBS suspension using this technique (Lu et al. 2010). In the presence of ErbB2-positive cells, the two-photon scattering intensity increased compared to the ErbB2-negative cell line. Lu *et al.* (2010) also demonstrated that the scattering-based system was two orders of magnitude more sensitive (100cells/ml) than the colourimetric equivalent in detecting ErbB2-positive cancer cells.

Nanoparticles in detection of soluble ErbB2 and circulating tumour cells

The electroconductivity of gold NMs has also been exploited to create an electrochemical detection system for sErbB2. Mucelli *et al.* (2008) demonstrated the detection of ErbB2 in SKBR-3 and MCF7 cell lysate preparations using Trastuzumab-functionalised gold nanoelectrodes which generate an electrocatalytic signal upon analyte binding. Although the detection system showed successful detection of ErbB2 in a complex biological solution, the detection limits attained by this setup were not improved over current ELISA detection limits. The detection of CTCs using nanomaterial-based technologies, as demonstrated in the CTC

model by Lu *et al* (2010), has been met with some success and a NP-based system has been commercialised (CellSearch®, Janssen Diagnostics, Inc., Belgium) which uses functionalised magnetic nanoparticles to separate CTCs from whole blood. Ghazani *et al* (2012) have demonstrated the detection of CTCs based on a combined four-marker biomarker set (Epithelial Cell Adhesion Molecule, ErbB2, ErbB1 and Mucin-1). This multiplexed approach uses micro-NMRI to detect functionalised iron oxide nanoparticles in a complex blood sample, without the need for primary CTC isolation. This offers greater chance of capturing CTCs and illustrates how measuring multiple targets may increase the diagnostic predictive value of a clinical *in vitro* diagnostic.

1.4 Nanoparticle applications for *in vivo* ErbB2 imaging

In vivo imaging techniques allow the assessment of internal anatomical structures. Techniques such as X-ray computed tomography (XRCT) (section 3.3.2) and Magnetic resonance imaging (MRI) (section 3.3.3) have become invaluable as medical assessment and diagnostic tools. More recently, near-infrared (NIR) spectrum contrast agents have been demonstrated as suitable superficial tissue contrast agents (De Grand & Frangioni 2003). Despite the advances made towards *in vivo* imaging, there are a number of aspects associated with these imaging modalities which need to be improved upon (e.g. administered contrast agent dose, short renal clearance times, non-specific distribution of contrast agents and reliance on the EPR effect alone for the visualisation of tumours). Due to their size, composition, and functionalisation potential, some NPs are suitably positioned to provide answers to the current *in vivo* imaging needs. NMs can be tuned to achieve desired renal clearance times and so provide a concrete opportunity for multimodal imaging of malignancies (Doane & Burda 2012). NMs can naturally accumulate in tumours due to the Enhanced Permeability and Retention (EPR) effect (a phenomenon observed in solid tumours where molecules of certain sizes tend to accumulate more in the tumour tissue in comparison to the normal tissue), and this quality may be enhanced through NM functionalisation in order to increase the sensitivity and specificity of ErbB2 tumour detection. The following section (summarised in Table 1.7) illustrates the application of nanotechnologies towards the enhancement of *in vivo* ErbB2 cancer detection.

When developing NPs such as SPIONs, polystyrene beads, noble metal NPs and QDs for *in vivo* applications (see table 1.7), cytotoxicity and biocompatibility issues must be considered. Many of these particles have been incorporated into liposomes for a variety of different applications to best maximise the delivery of localised drug or contrast imaging enhancer where necessary (Schütz *et al.* 2013). In this respect, only those particles that have already been assessed or evaluated in their associated risk for *in vivo* imaging are considered. Over

the past 10 years, iron oxide nanoparticle formulations have been brought through pre-clinical and clinical evaluation and are available as FDA- or EMA-approved MRI contrast agents, such as Endorem® (Feridex IV) and Resovist® (Cliavist®). Therefore, in the case of SPIONs, cytotoxicity and biocompatibility have been taken into account during the pre-clinical and clinical phase I trials. Currently, a number of EU FP7 projects (such as the MULTIFUN project) are engaged in the development of a new generation multifunctional SPION for theranostic application for *in vivo* applications (Prina-Mello et al. 2013). Polystyrene (PS) beads have been extensively used as a biocompatible multimodal system for enhanced imaging. PS beads have been proven to have several advantages from their controllable sizes to their tunable functional responses with the surrounding tissue (Sánchez-Martín et al. 2008). Au and Ag have been extensively used for their plasmonic imaging properties and antibacterial properties (Seil & Webster 2012), respectively. The biocompatibility of Ag NPs is illustrated by several products on the market including bandages, plasters, tiles, and paints which all work on the principle of nano-Ag antibacterial principle. The “nano-” formulations of gold may be advantageous for tackling very specific disease and healthcare applications, and gold NP formulations such as Aurimmune® are currently in phase II trials for solid tumour treatment (Etheridge et al. 2013). That aside, there are at least 5 Au NP products on the market commercially available which have been approved for cancer imaging and theranostic purposes (Jain et al. 2012).

In the past 10 years, QDs have been proven to be the future alternative to colorimetric dyes due to their intrinsic properties. Several studies have also provided evidence for *in vivo* applications (Tiwari Dhermendra K et al. 2011, Yang et al. 2012). The speculations in this respect are twofold: 1) QDs, if properly coated, can be made biocompatible and non-cytotoxic and 2) the efficacy of real-time QD imaging *in vivo* in life-threatening (or irreversible) disease can overcome the localised biocompatible limitation of the QD core (in the eventuality that this will be fully exposed to biological tissue/fluid).

Furthermore, in all nanomaterials here presented, there is growing evidence from the physico-chemical applications that these can be designed (or assembled) into a multi-layer configuration to reduce, prevent or neutralise any possible adverse aspect derived from the interaction with the *in vivo* biological tissue or fluid (Movia et al. 2013). This is particularly true for iron oxide, Ag and Au NPs due to either the low toxicity or inert nature (Dreaden et al. 2012, Veisheh et al. 2010).

Table 1.8 Nanoparticle applications in *in vivo* detection and imaging of ErbB2 in tissue. The technical details section gives information on the nanoparticle format and application, as well as the declared sensitivity of each system if mentioned. The approaches are ranked in terms of their potential to affect clinical translatability. Clinical translatability was assessed principally under ability to specifically detect ErbB2, comparison to conventional techniques, improved aspects/limitations, sample type examined and ease of use.

Technical Details					Clinical Translation Details			
Tissue ErbB2 form detected	Detection/Imaging method [†]	Nanoparticle Format	Sample tested*	Sensitivity declared	Improvement over existing technology [‡]	Limitations with nanoparticle product	Score [∞] 1-5	Reference
Cell surface ErbB2	SQUID sensor detection	SPION coupled to antibody	Cell lines MCF7, BT-474, and MDA-MB-231	940,000 cells at 4.5cm depth	Binding-dependent, ErbB2-targeted magnetic contrast agent.	<i>In vivo</i> toxicity and targeting not assessed.	4	Hathaway et al 2011
	Fluorescence microscopy	Trastuzumab-polystyrene NPs doped with cyanine Itrybe	Tumour xenograft and subcutaneous injection.	Cell type specific detection of ErbB2	Superior NIR emission properties vs organic dyes. Low dose needed for dye visualisation compared to existing dyes.	Imaging of tumour <i>in vivo</i> not demonstrated.	4	Behnke et al 2013.
	SERS	Noble Metal nanoparticle coupled to antibody	Cancer xenograft		NIR active Raman reporter. Deep tissue penetration. Improved Raman signal vs conventional NIR agent.	Biodistribution of nanoparticle not assessed.	4	Samanta et al 2011
	Fluorescent imaging and MRI	Affibody-QD and Affibody-SPION	Tumour xenograft imaging		Targetable & improved magnetic contrast vs conventional agent.	Sequestration of particles to non-tumour organs.	3	Gao et al 2011
	MRI	SPION	ErbB2-		Targetable MRI contrast agent.	Sequestration of particles	3	Kievit et al 2012

		coupled to antibody	transgenic mouse model			to non-tumour organs.		
MRI and SQUID		SPION coupled to antibody	Cell line MCF7/Her2-18	104,000 cells/ml via MRI	Multimodal detection. Defined detection limits. Functionalised magnetic contrast agent.	Comparison to conventional techniques not shown. Medium ease of use.	3	Adolph et al 2012
SERS		Noble Metal nanoparticle coupled to antibody	Cell line SKBR-3	Not reported	Subcutaneous detection of the nanoparticle.	SKBR-3 cells were pre-tagged with nanoparticle prior to injection rather than NP injection alone followed by tumour tracking.	2	Maiti et al 2010
X-ray CT		Noble Metal nanoparticle coupled to antibody	Cancer xenograft	Not reported	Detection of small tumours	Comparison to conventional techniques not shown. Low ease of use. Non-specific uptake.	2	Hainfeld et al 2011
†SQUID: Superconducting Quantum Interference Device; SERS: Surface-enhanced Raman spectroscopy; MRI: Magnetic Resonance Imaging; CT: Computed Tomography. ° NIR: Near Infrared.								

1.4.1 Optical imaging of ErbB2 protein *in vivo* using near-infrared-emitting nanoparticles

In vivo optical imaging techniques which attempt to use light emitted in the visible spectrum (~390-700 nm wave length) have limited penetration depth in tissues. This is due to the strong tissue auto-fluorescence under external excitation and the scattering and absorption of short-wavelength light by tissues and the water contained within tissue (Chuang et al. 2014). However, materials which emit light in the NIR spectrum (~700-1600nm wavelength radiation) have particular advantages for *in vivo* molecular imaging due to the fact that these wavelengths are minimally absorbed by body tissues (Hilderbrand & Weissleder 2010, Yi et al. 2014). Optical imaging at NIR wavelengths can extend the tissue penetration depth (i.e. the depth at which NIR radiation can be emitted which allows capture of that radiation for analysis by a fluorescent detector) up to several centimetres in some cases (Chen et al 2012). To date many organic NIR imaging probes have been limited in clinical use due to aqueous insolubility, aggregate formation and small Stokes shifts (Escobedo et al. 2010). The ability of QDs and other NPs to emit efficiently in the NIR makes them promising candidates as NIR contrast agents and have focused attention on the development of NM-based probes for subcutaneous *in vivo* imaging (Hong et al. 2009, Kim et al. 2004). Issues surrounding the *in vivo* use of QDs such as long term *in vivo* distribution, clearance, and toxicity of QDs need to be characterised (Yong et al. 2009) before they can be deemed suitable for optical imaging as an *in vivo* diagnostic tool. The absorption by tissue of the excitation radiation is also a factor to consider, which may limit the utility of a potential *in vivo* optical imaging technique, though recent work involving photostimulated persistent luminescent NPs may be one way of addressing this limitation (Chuang et al. 2014). Though several examples have demonstrated the potential use of NPs in NIR deep tissue imaging (Chen et al. 2012, Chuang et al. 2014), it remains to be seen whether these NPs can overcome issues such as non-specific bio-distribution and toxicity.

For example, Gao et al (2011) coupled an anti-ErbB2 affibody to the surface of QDs, followed by systemic probe injection and imaging of human SKOV-3 tumours in immune compromised mice. NIR fluorescence (NIRF) *in vivo* imaging showed that functionalised anti-ErbB2 QDs selectively bound to and accumulated in SKOV-3 tumours compared to non-functionalised PEG-coated QDs. Lately, Trastuzumab-functionalised polystyrene NPs containing NIR-emitting heptamethine dye have been developed to facilitate *in vivo* imaging of ErbB2-positive tumours in mice (Behnke et al. 2013). These particles have shown to successfully detect ErbB2 in cancer cell lines and fixed tumour tissues. *In vivo*, the same NPs could be visualised by NIRF imaging when injected subcutaneously into mice, leading to the possibility of use as future *in vivo* imaging probes.

There are still a number of obstacles to overcome before QDs or other NPs become commonplace in *in vivo* diagnostic and clinical practise. Although NIR QDs would be useful *in vivo* imaging of superficial cancers, their application is depth-limited to not more than 2-3 cm (Chen et al. 2012, Chuang et al. 2014). In addition, the cytotoxicity of QDs (Austin et al. 2004) and ability to selectively target tumour tissue still need to be fully addressed. Due to their sequestration in non-cancerous organs, QDs will most likely not be systemically administered, but administered directly to the tissue of interest. Parameters such as NP size, composition, surface functionalisation and administered dose all contribute to whether NPs will be used *in vivo*. Despite possessing excellent fluorescent properties (such as tuneable NIR spectrum emission) there is still a way to go before QDs will be considered safe for *in vivo* imaging of cancers.

A relatively new and exciting NP-based *in vivo* imaging technology has emerged from NPs which exhibit the phenomenon of second harmonic generation (SHG). SHG occurs when mono-chromatic electromagnetic radiation is passed through an optically non-linear material and generates an output wave with double the frequency to that of the incident radiation. This phenomenon is a property of materials with a non-centrosymmetric crystal lattice structure, and can be found in nanomaterials such as CNTs, ZnO and GaN nanowires, and nanocrystals such as BiTiO₃ (Suresh & Arivuoli 2012). Although SHG is susceptible to depth-restriction in tissue imaging, a potential advantage of using these NMs would be that the incident radiation could be selected so that the emission radiation (at double the initial frequency) would be minimally absorbed by tissue. This may limit the interference of tissue with the emission radiation. Additionally, SHG is not restricted by an absorption/excitation spectra window and is not prone to bleaching or blinking effects (Bäumner et al. 2010). These characteristics make particles which possess SHG potentially ideal for deep tissue *in vivo* imaging through measurement of coherent interference from NP populations. Although ErbB2 imaging *in vivo* using SHG has not been demonstrated yet, it is an avenue of research which looks very promising for future clinical applications, especially in imaging biopsies.

1.4.2 X-ray Computed Tomography (XRCT) of ErbB2 tumours using metal nanoparticles

XRCT, also known as Computed Tomography (CT) or Computed Axial Tomography (CAT) images *in vivo* architecture based on obstruction of X-radiation by tissue structures (Kalender 2006). Contrast agents (such as iodine) are used to enhance radiopacity for imaging purposes (Figure 1.8); however short renal clearance times of such contrast agents require high doses to be administered, thus raising toxicity issues. Loading tumour marker antibodies with CT

contrast agents has attempted to reduce the administered dose needed but insufficient Iodine loading on the antibody resulted in suboptimal CT conditions (Hainfeld et al. 2011).

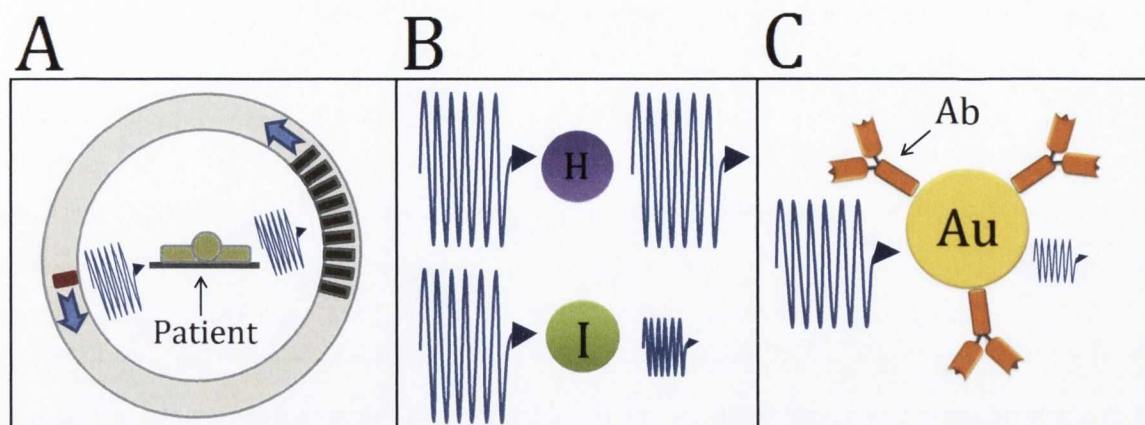


Figure 1.10 Principle of X-Ray Computed Tomography. (A) Schematic of XRCT in a clinical setting; X-rays (blue oscillating wave) pass through the patient's body and are detected on the opposite side (brown rectangles) to the X-ray generator (red). The attenuated X-rays collected by the detector are converted into a digital signal for image processing. (B) The molecular underpinnings behind XRCT; In the body, atoms such as Hydrogen (H) do not attenuate X-rays whilst Iodine (I) contained within contrast agents measurably block X-rays. (C) The potential use of Au NPs in XRCT. Au NPs (yellow) readily block X-rays and can be tumour targeted by functionalising the Au NP surface with antibodies (Ab).

Several noble metal and non-noble metal NPs (namely bismuth (Bi) and ytterbium (Yb) NPs) with high radiopacity have been developed as CT contrast agents in an attempt to target and improve image contrast in *in vivo* CT imaging (Liu et al. 2012). The main advantages of using nanotechnology-based contrast agents include tuneable blood half-life, and a high X-ray attenuation coefficient as compared to iodine, making it useful as a potential contrast agent (Cai et al. 2007).

In a proof of principle study, Trastuzumab-functionalised Au NPs were used to detect ErbB2-positive cancer xenografts in mice using Micro-CT (Hainfeld et al. 2011). This study demonstrated accumulation of anti-ErbB2 Au NPs to the tumour site and detection of small tumours (1.5mm). However, significant nonspecific accumulation of unconjugated Au NPs in ErbB2-positive and ErbB2-negative tumours (possibly due to EPR effect) appears to limit their use as ErbB2-specific *in vivo* targeting tools.

1.4.3 Magnetic imaging of ErbB2 tumours using magnetic nanoparticles

For many years, MRI (which measures proton density and relaxation rate in a magnetic field) has been used for the non-invasive, three-dimensional imaging of *in vivo* tumours (Morris

2002) (Figure 1.9). Conventional MRI relies on a pre-contrast and post-contrast image in order to locate and define the tumour from the high resolution background image (Lemort et al. 2007) and requires significant anatomical knowledge on behalf of the analyst in order to identify the tumour relative to surrounding tissue.

In order to enhance tumour image contrast, efforts have been made to couple contrast agents such as radioactive elements (Sampath et al. 2007) to anti-ErbB2 ligands in an attempt to improve the detection of ErbB2-positive cancer. Still the low biocompatibility of these contrast agents has spurred researchers to investigate novel nanotechnology-based magnetic labels. Superparamagnetic iron oxide nanoparticles (SPIONs) are one of the main categories of NMs used as magnetic contrast agents. Their surface can be functionalised with bioactive marker ligands such as antibodies and can be coated with biocompatible materials such as silica (Laurent & Mahmoudi 2011). These non-radioactive, biocompatible SPIONs have been used to detect ErbB2 *in vitro* (Yang et al. 2010) and can be used to separate ErbB2-positive SKBR-3 cells from ErbB2-negative NIH/3T3 cells in an *in vitro* model for cancer cell sorting (Mi et al. 2011). However, their real value will probably be found in *in vivo* applications where deep penetrating tissue contrast agents are necessary. The potential of SPIONs as valuable *in vivo* magnetic contrast agents has been demonstrated in murine tumour xenograft explants (Hathaway et al. 2011), where anti-ErbB2-conjugated SPIONs injected into ErbB2-expressing xenografts yielded higher magnetic response via superconducting quantum interference device (SQUID), compared to unconjugated SPIONs. MRI of anti-ErbB2-conjugated SPIONs in a non-immune-compromised ErbB2-transgenic mouse model (Kievit et al. 2012) demonstrated significant shortening in T_2 magnetic relaxation time in the tumour region compared to no injection, and suggests that these SPIONs can provide contrast enhancement in tumour tissue. Although the decrease in T_2 was larger in anti-ErbB2 SPIONs compared to IgG-conjugated SPIONs, the difference was not statistically significant at the low sample number used ($n=3$).

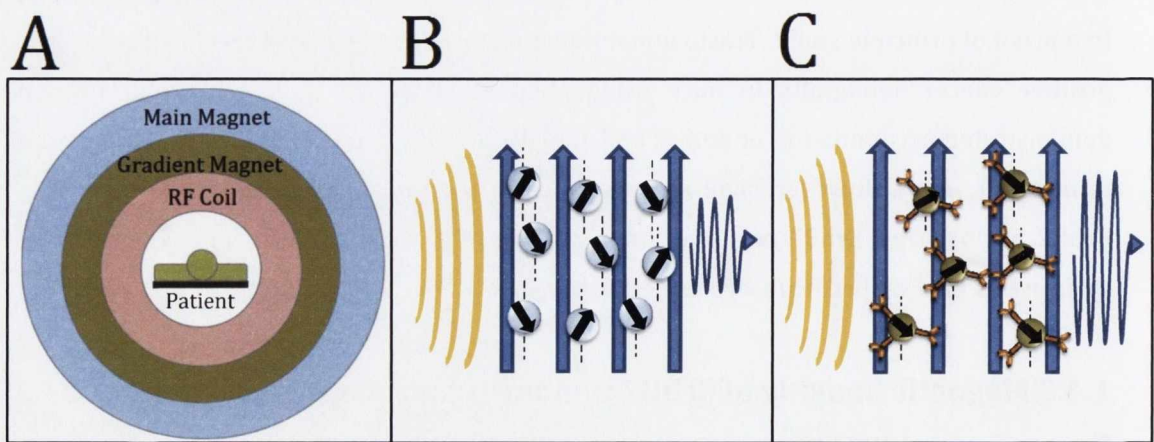


Figure 1.11 Principle of Magnetic Resonance Imaging. (A) Schematic of MRI in a clinical setting: The main magnet and gradient magnet are used to align the atomic nuclei within the patient's body. (B) The molecular underpinnings behind MRI: The radio frequency (RF) coil is used to alter atomic alignment and a magnetic moment is released from the atoms as they relax to their normal magnetic alignment. (C) The use of SPIONs in MRI. SPIONs generate a large magnetic moment when returning to their normal magnetic alignment. The attached antibodies (orange) will allow targeting of SPIONs to tumour tissue. Key: Yellow bands: radio frequency; Vertical blue arrows: magnetic field; Oscillating blue arrow: released energy from relaxing proton/superparamagnetic NP.

More recently, magnetic relaxometry of SPIONs measured by SQUID offer the chance to significantly improve *in vivo* imaging of tumour tissue over MRI imaging. SQUID sensors are tuned to only capture the magnetic field relaxation of target-bound NPs, thus eliminating background contrast interference. SQUID measurements improve therefore the magnetic signal-to-noise ratio of the tumour but lose the context of tumour positioning within tissue (Flynn & Bryant 2005). This deficit can be overcome by integrating SQUID measurements with SQUID-detected ultra-low-field-MRI. Hathaway *et al.* (2011) used SQUID sensors to detect ErbB2-positive tumour explants using anti-ErbB2 conjugated SPIONs. Notably, these nanotechnology-based probes allowed the detection of low numbers of labelled tumour cells within breast phantom tissue at a depth of nearly 5 centimetres. Commercially available SPIONs (Endorem®) are being trialled for the SQUID-based magnetic detection of cancerous sentinel lymph nodes in breast cancer patients (Johnson *et al.* 2010).

Direct comparison of SPIONs detection of ErbB2-positive cancers by MRI and magnetic relaxometry showed that both techniques are able to detect and image MCF7/Her2-18 tumour xenografts in mice. High sensitivity in tumour detection was possible by SQUID detection of targeted SPIONs (Adolphi *et al.* 2012), however the lack of comparison to ErbB2-negative tumour xenografts means that the specificity of the functionalised SPIONs was not fully tested.

With the ability to functionalise their surfaces, magnetic NPs are set to become the magnetic contrast agents of the near future, providing targetable, non-radioactive imaging tools for specific cancer subtypes. SQUID sensor developments, as well as other advantages of relaxometry (such as linear magnetic moment relative to NP number) may provide additional sensitivity for the generation of high quality imaging tools to diagnose tumours. However, as with the use of other NPs *in vivo*, a significant consideration is the sequestration of SPIONs by non-cancerous organs such as the liver and kidneys. These issues may be addressed through NP engineering approaches and surface functionalisation.

1.5 Conclusions

Clinically, ErbB2 overexpression is considered to be an important marker for a number of solitary and metastatic cancers and sErbB2 is recognised as a promising blood biomarker in profiling ErbB2-positive cancer progression. Although current clinical diagnostic approaches give the clinician a reasonable assessment of ErbB2 load within a patient, this review highlights the inherent limitations of these systems and underscores the need for new technologies which aim to bringing clinical diagnostics to new levels of molecular specificity and sensitivity.

The option to functionalise NPs with ligands has the potential to improve clinical diagnosis of ErbB2 with respect to sensitivity, objectivity and multiplexing of biomarker analysis for clinical diagnostic applications. Although currently, NM-based technologies may sometimes suffer from limitations such as batch-to-batch reproducibility (Nightingale & de Mello 2010), it is expected that with constantly improving understanding of NP synthesis and functionalisation, the advantages of NP-based diagnostics will be fully realisable.

In vitro, NMs such as QDs provide a real opportunity to improve the quality of ErbB2 diagnostics, particularly in protein quantification assays. QDs have been used to improve the sensitivity and objectivity of a routine ErbB2 IHC diagnostic, for a more robust disease staging. QD use in FACS has the potential to simplify multiplexed cancer assays, thus increasing the confidence which clinicians can place in the diagnostic readout. Additionally, noble metal NP application to SERS-based and electroconductive ErbB2 detection systems is likely to enhance future *in vitro* cancer diagnostic kits. The availability of functionalised SPIONs as a commercially viable diagnostic product (CellSearch®) for simplified CTC detection in complex patient sample, illustrates the benefits that NMs can bring to improving the simplicity and specificity of *in vitro* ErbB2 cancer diagnostic assays.

In vivo, NPs are suitably positioned to meet medical demands for targeted imaging of cancers. The ability of QDs to emit efficiently in the NIR makes them promising NIR contrast agents and has focused attention on the development of QD for subcutaneous *in vivo* imaging. Other NMs which possess SHG may be ideal for deep tissue *in vivo* cancer imaging. In terms of enhancing existing *in vivo* cancer imaging techniques, several functionalised noble metal NMs have been developed in an attempt to target and improve image contrast in XRCT imaging. This NP application has achieved detection of small tumour populations, though issues of nonspecific accumulation of unconjugated Au NPs in ErbB2-positive and ErbB2-negative

tumours appears to limit their widespread application for ErbB2-specific tumour detection for the time being.

SPIONs are a category of NMs which are already commercially available for use as *in vivo* magnetic contrast agents and it is hoped that this NM type will be further refined to provide cancer-targetable imaging tools for specific cancer subtypes such as ErbB2, while reducing the dose administered to the patient. SPION development, along with magnetic sensor improvements (such as SQUID), may provide additional sensitivity for the generation of high quality detection tools to diagnose ErbB2-specific tumours in depth.

To conclude, limitations in current *in vitro* and *in vivo* ErbB2 diagnostic approaches are hindering the advancement of cancer treatments against this often-aggressive molecular subtype. The development of more reliable methods to assess ErbB2 load and activity in tissue and blood fractions will inevitably help to improve the quality of treatment which cancer patients receive. To this end, there is a demand for *in vitro* and *in vivo* ErbB2 assays with increased sensitivity, specificity and multiplexed ability. A range of NMs have been developed to meet some of these medical needs, and the current and potential benefits of NM-based ErbB2 assays have been illustrated. From the assessment of the current scientific and technological developments surrounding ErbB2 detection, it is expected that in the next five to ten years several of the pre-clinical NM-based techniques and methodologies described are set to be translated into practise for the benefit of the patients, clinicians and healthcare providers.

1.6 Thesis Aims

The main aim of this body of work is to develop a nanowire-based platform for the detection of multiple cancer biomarkers in complex biological samples. This aim is sub-divided into several goals:

- Conjugate a cancer biomarker-specific antibody to the surface of a nanowire
- Validate that the antibody is stably attached to the nanowire

- Assess whether the antibody is biologically active on the nanowire. Can it detect the target biomarker in a simple biological system?
- Assess whether the nanowire assay can detect the target biomarker in a complex biological medium
- Develop novel technological approaches to help in the incorporation of nanowires into future diagnostic platforms

The work presented in the following chapters will aim to address these aims.

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Chapter 2

Materials and Methods

2.1 Materials

Table 2.1 Materials

Material	Catalogue Number	Supplier
Anti-CD340-AlexaFluor488	324402	Biologend
Anti-Rabbit IgG, HRP-linked	7074	Cell Signalling
Anti-mouse IgG, HRP-linked	7076	Cell Signalling
ErbB2 (29D8) Rabbit mAb	2165	Cell Signalling
Fluorescent Mounting Medium	S3023	Dako
Chromatography Paper	ST02-Y001	Fisher
Phosphate Buffered Saline	10010	Gibco
Flow cytometry size calibration kit	F-13838	Invitrogen
See Blue Plus2 Prestained Standard	LC5925	Invitrogen
X-Omat LS Film	F1274	Kodak
Trypan Blue	T10282	Life Technologies
TrypLE™ Select	12563-029	Life Technologies
Trail Mix™ HRP Western Blot Markers	70982	Novagen
S-Protein HRP Conjugate	69047	Novagen
Lab-Tek® chamber slides	177445	Nunc
Universal Casein Diluent	UCDB	SDT
Agarose	A9539	Sigma-Aldrich
Anti-β-Actin mouse mAb	A2228	Sigma-Aldrich
Ammonium Persulfate	A3678	Sigma-Aldrich
Bicine	B3876	Sigma-Aldrich
Bis-Tris	B9754	Sigma-Aldrich
Bradford Reagent	B6916	Sigma-Aldrich
Bromophenol Blue	B0126	Sigma-Aldrich
Chlorobutanol	112054	Sigma-Aldrich
Dithiotreitol	D0632	Sigma-Aldrich
Ethylenediaminetetraacetic acid (EDTA)	EDS	Sigma-Aldrich
Formaldehyde solution 36.5%	F8775	Sigma-Aldrich
HEPES	H3375	Sigma-Aldrich
Glycerol	G5516	Sigma-Aldrich
Glycine	G8898	Sigma-Aldrich
IGEPAL 630	I7771	Sigma-Aldrich
Methanol	65542	Sigma-Aldrich
β-Mercaptoethanol	M6250	Sigma-Aldrich

NaF	S7920	Sigma-Aldrich
Na ₃ VO ₄	S6508	Sigma-Aldrich
Polyvinylidene difluoride membrane	P2813	Sigma-Aldrich
Phenylmethanesulfonyl Fluoride	P7626	Sigma-Aldrich
Sodium Chloride	S7653	Sigma-Aldrich
Sodium Dodecyl Sulfate	L3771	Sigma-Aldrich
Sodium Hydroxide	S8045	Sigma-Aldrich
Tetramethylethylenediamine	T9281	Sigma-Aldrich
Tris-Base	T1503	Sigma-Aldrich
Tris-HCl	T3253	Sigma-Aldrich
Tween 20	P2287	Sigma-Aldrich
Albumin Standard	23209	Thermo Scientific
Hoechst Fluorescent Stain	33342	Thermo Scientific
Acrylogel 2.6 (30%) Solution Electran®	427205E	VWR
Boric Acid	BDH9222	VWR
Butanol	10061	VWR
Coag-Norm ® Plasma	00522	Diagnostica Stago

2.2 Methods for Chapter 3

2.2.1 Cell culture

Six cell lines were used for antibody and nanoprobe validation experiments. Cell lines were chosen from the American Type Culture Collection (ATCC) biological resource centre catalogue (VA, USA) and were selected to reflect a range of target protein expression levels. Three primary epithelial breast cancer-derived cell lines (BT474, SKBR3 and MDA-MDB-231) and three epithelial lung cancer-derived cell lines (A549, H596, H520) were selected. All cell lines were maintained between passage 3 and 20 in basal media containing 1% Penicillin/Streptomycin supplemented with Fetal Bovine Serum (FBS, South American origin, Gibco, USA) (Table 1).

Table 2.2 Cancer-derived cell lines for use in *in vitro* antibody testing.

Cell line	Basal Medium†	Serum Supplement*	Cancer type	Her2 Expression level (Bunn et al. 2001, Subik et al. 2010)
BT-474	RPMI 1640	10% FBS, 1% P/S, 1% SP	Ductal carcinoma	3+
SK-BR-3		10% FBS, 1% P/S	Adenocarcinoma	3+
MDA-MB- 231	DMEM		Adenocarcinoma	1+
A549	F-12 Ham I		Carcinoma	2+
H520	RPMI 1640		Squamous cell carcinoma	0
H596		20% FBS, 1% P/S	Adenosquamous carcinoma	1+

* FBS: Fetal Bovine Serum; P/S: Penicillin/Streptomycin; SP: Sodium Pyruvate. †RPMI: Roswell Park Memorial Institute Medium; DMEM: Dulbecco's Modified Eagle Medium.

2.2.2 Morphological imaging of cell lines

Morphological images for cell lines were obtained using a brightfield microscope (Zeiss, Germany) at 20X or 40X magnification. Minimal image enhancements (brightness and

contrast adjustment) were made using ImageJ software (National Institutes of Health, USA).

2.2.3 Assessment of performance of ErbB2 antibody for use in nanoprobe platform

Two ErbB2 antibodies were used in the initial proof-of-concept platform, namely Trastuzumab and a commercially available fluorescently-labelled ErbB2 antibody (anti-CD340-AlexaFluor488: Biolegend, US). The specificity of Trastuzumab is well documented as it is in therapeutic use as an anti-ErbB2 monoclonal antibody. The specificity of anti-CD340-AlexaFluor488 for ErbB2 was unknown and examined by staining cancer cell lines expressing varying levels of cell surface expression of ErbB2 (previously characterised by Western Blotting and ELISA prior to testing). Antibody performance assessment was carried out via fluorescent confocal microscopy.

2.2.3.1 Western Blotting assessment of total ErbB2 protein in cell lines

Western blotting was used to confirm the presence of ErbB2 protein in each cell line.

Whole cell protein extraction

Cells (BT-474, SK-BR-3, MDA-MB-231, A549, H520, H596) were cultivated for 72 hours and lysates were harvested at 80-90% cell confluence. Briefly, cells were washed once with sterile PBS. All following steps were performed on ice where possible. Cells were scraped into solution and pelleted under centrifugation (PrismR, Labnet International Inc., Edison, NJ, USA) at 2000 g for 5 min at 4 °C. The pellet was resuspended in 50 µl lysis buffer (20 mM HEPES pH7.6, 400 mM Sodium Chloride, 1 mM EDTA pH8, 5 mM NaF, Na₃VO₄, 25% Glycerol, 0.1% NP40 (IGEPAL 630), 1 mM Phenylmethanesulfonyl Fluoride, 1 mM Dithiotreitol) containing protease inhibitors (Complete protease inhibitor cocktail, #1697498, Roche) and incubated on ice for 10 min. Cell membranes were passed through a 25G needle (Neolus, Terumo, Belgium) 15 times and incubated on ice for 30 min. Lysates were placed under centrifugation for 10 min at 17,200 g at 4°C in order to remove remaining cell debris. The supernatant containing total protein extract was stored at -80°C.

Total protein quantification

Total protein content of harvested cell lysate was determined using Bradford Reagent (#B6916, Sigma-Aldrich) as per manufacturer's instructions. Sample protein quantification through colourimetric measurement at 495nm (Epoch, BioTek®) was made through comparison to a standard curve of serially diluted Albumin standard.

Protein separation using acrylamide gel platform

The resolving gel (8%) and stacking gel (4%) were prepared as in Table 2.

Table 2.3 Preparation of Stacking and Resolving acrylamide gels for protein separation

Reagent	Stacking Gel 4%	Resolving Gel 8%	Resolving Gel X%
30% Acrylamide/Bis 37.5 : 1	660 µl	2.64 ml	0.33 * (X%) = (A) ml
1M Tris-HCl pH 6.8	630 µl	---	---
1.5M Tris-HCl pH 8.8	---	2.5 ml	2.5 ml
10% Sodium Dodecyl Sulfate	50 µl	100 µl	100 µl
Distilled Deionised H ₂ O	3.63 ml	4.71 ml	7.35 - (A) ml
10% Ammonium Persulfate	25 µl	50 µl	50 µl
TEMED	5 µl	5 µl	5 µl

Protein samples were prepared to a final working concentration of 3 mg/ml containing 1X loading sample buffer (SDS 3% (w/v), glycerol 30% (v/v), β-Mercaptoethanol 3% (v/v) and bromophenol blue 0.005% (w/v)). Due to prior experience that BT-474 and SK-BR-3 cell lines with very high expressers of ErbB2, a further 1:30 dilution of lysate was made before loading to the gel. This is reflected in the results section. Samples were denatured at 98 °C for 2.5 min before immediate loading (20 µl) to the gel in a vertical Mini Slab electrophoresis rig (AE-6450, Atto, Japan). Molecular ladders (Table 2.7) See Blue Plus2 Prestained Standard (10 µl) and Trail Mix™ HRP Western Blot Markers (10 µl) were loaded to enable subsequent protein sizing. The gel was placed under electrophoresis in 1X running buffer (Table 2.4) at 35mA (up to max 200mV) until the leading phase of the marker had run off the gel.

Table 2.4 10X Running Buffer

Tris-Base	30.28 gr
Glycine	144.25 gr
10% Sodium Dodecyl Sulfate	10 gr
H ₂ O	To 1L

Table 2.5 Transfer Buffer

Tris-Base	5.8 gr
Glycine	29 gr
Sodium Dodecyl Sulfate	1 gr
Methanol	200 ml
H ₂ O	To 1 L

Table 2.6 1X TBST

1M Tris-HCl	20 ml
5M NaCl	30 ml
Tween 20	1 ml
H ₂ O	To 1 L

Transfer of proteins to PVDF membrane using semi-dry transfer method

Electrophoresed proteins and size ladders were transferred to a methanol-activated polyvinylidene difluoride (PVDF) membrane using a semi-dry transfer technique (Figure 2.1) containing transfer buffer (Table 2.5) in a horizontal electrode plate rig (Horizblot, AE-6675, Atto, Japan) for 2.5 hr at 250 mA. The PVDF membrane was stored in Tris-buffer Saline Tween 20 (TBST) buffer (Table 2.6) at 4 °C until staining.

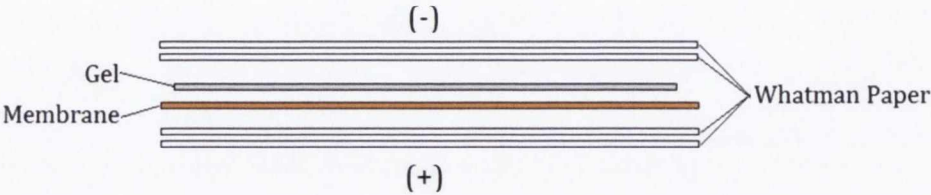


Figure 2.1 Assembly of the stack for semi-dry transfer of proteins. The transfer stack was orientated between the rig cathode (-) and anode (+) as shown.

Western blot detection of ErbB2 protein

ErbB2 and β -Actin was detected using primary antibody (Table 2.7), amplified by horseradish peroxidase-linked secondary antibody and visualised by exposure to photosensitive film in the presence of a chemiluminescent substrate.

Table 2.7 Antibody and Molecular Size Ladder component list for western blotting protocol

Material	Catalogue Number	Supplier
Anti-Rabbit IgG, HRP-linked	7074	Cell Signalling
Anti-mouse IgG, HRP-linked	7076	Cell Signalling
ErbB2 (29D8) Rabbit mAb	2165	Cell Signalling
Trail Mix™ HRP Western Blot Markers	70982	Novagen
S-Protein HRP Conjugate	69047	Novagen
Anti- β -Actin mouse mAb	A2228	Sigma-Aldrich

The PVDF membrane was incubated with ErbB2 Rabbit monoclonal antibody diluted (1:2000) in TBST with 10% (w/v) dried skimmed milk (Marvel) for 2 hr at room temperature. Excess antibody was removed by washing with TBST buffer and incubated with HRP-linked Anti-Rabbit IgG secondary antibody (1:3000) and HRP-linked S-Protein (1:5000) for 1 hr at room temperature. Excess antibody was removed by washing with TBST buffer.

Exposing membrane

The PVDF membrane was exposed for 5 min with Forte substrate (Millipore, USA). Chemiluminescence was visualised in the dark room through exposure (15 sec) of X-Omat LS Film (Kodak, USA). Protein sizes were estimated through comparison to size ladders.

Western blot detection of Actin protein

When reprobing the same membrane for Actin, the PVDF membrane was stripped of antibody using Antibody Stripping Solution (Millipore, USA) as per the manufacturer's instructions. The PVDF membrane was incubated with Anti- β -Actin mouse monoclonal antibody diluted (1:4000) in TBST with 10% (w/v) dried skimmed milk for 1 hr at room temperature with constant rocking. Excess antibody was removed by washing with TBST buffer and the membrane was incubated with HRP-linked Anti-Mouse IgG secondary antibody (1:3000) and HRP-linked S-Protein (1:5000) in TBST with 10% (w/v) dried skimmed milk for 1 hr at room temperature on a rocker. Excess antibody was removed by washing with TBST buffer and the membrane was exposed as before.

2.2.3.2 ELISA quantification of ErbB2 protein in whole cell lysate

Whole cell protein extraction and total protein quantification was performed as previously stated (section 2.2.3.1). ErbB2 protein was measured in the whole cell lysate of cell lines using the human ErbB2 DuoSet® ELISA Development System (#DY1129, R&D Systems) as per the manufacturer's instructions, and normalised to total protein concentration. Final ErbB2 concentration was expressed as a fraction of total protein per sample and values were presented as mean $\pm$ SEM. Quantification of ErbB2 concentration was carried out in triplicate for each cell line and SKOV3 cell lysate was used as an ErbB2-positive control.

2.2.3.3 Fluorescent staining of cancer cell lines with fluorescent ErbB2 antibody

Cells were removed from the culture flask by enzymatic digestion (TrypLE™ Select) for 2 min at 37°C. Cells were resuspended in the relevant fresh media and counted via Trypan Blue Stain (0.4%) using the Countess® Automated Cell Counter (Invitrogen™, Life Technologies, USA). Cells were plated at 50,000 cell/ml on Permaxox® Lab-Tek® 8-well chamber slides (Nunc®, Denmark) and allowed to adhere overnight at 37 °C. Cells were fixed with 3.7% paraformaldehyde for 30 min at room temperature and washed three times (5 min) with PBS. Cells were stained with Hoechst nuclear stain (Thermo Scientific, USA) (1:800) and anti-CD340-AlexaFluor488 (Biolegend, USA) (1:500) for 2 hr at room temperature. Cells were washed three times (5 min) with PBS and mounted with a

coverslip in fluorescent mounting medium (S3023, Dako, Denmark). Imaging was done at 63X magnification with an oil immersion lens (Plan-Apochromat 63X/1.4) on a Zeiss confocal microscope system (Zeiss, Germany). Images were acquired with the multi-track (MT 405/488) setting and processed using Zeiss LSM Image Browser software (Zeiss, Germany). Images were enhanced with minimal use of brightness and contrast software controls and scale bars were added.

Table 2.8 Staining information and microscope settings for analysis of fluorescent staining of cancer cell lines

Staining information				Microscope Settings	
<u>Stain</u>	<u>Cellular Target</u>	<u>Peak Absorption Wavelength</u>	<u>Peak Emission Wavelength</u>	<u>Excitation wavelength (power)</u>	<u>Filter Set†</u>
<u>Hoechst 34580</u>	Nucleus	395nm	450nm	405nm (5%)	BP 420 – 480nm
<u>Anti-CD340-AlexaFluor488</u>	ErbB2	488nm	520nm	488nm (2.1%)	BP 505- 530nm
† BP: Band Pass					

2.2.4 Nanomaterials

2.2.4.1 Nanowires

Silica nanowires (synthesis and imaging: Gareth Clarke, TCD), Cobalt Nickel (CoNi) nanowires (synthesis and imaging: Dr Lorena Monzon, TCD) and Silver (Ag) nanowires (synthesis: Seashell Technology LLC, USA, imaging: Dr Joeseph McCarthy) were size characterised by analysis of scanning electron microscopy (SEM), transmission electron microscopy (TEM) or He-ion microscope images. Nanowire length distribution was measured using ImageJ software (n=30-100).

2.2.4.2 Nanospheres/Microspheres

Polystyrene flow cytometry calibration beads (F-13838) were obtained from Invitrogen and and size characterised using SEM (courtesy Ms. Laura Kickham, TCD). Silica nanospheres (diameter = 100nm) were obtained from Glantreo (Cork, Ireland) and size characterised using SEM and NanoSight NS500 (Wiltshire, UK) Nanoparticle Tracking Analysis (NTA) software.

2.2.4.3 Bulk nanomaterial imaging approach

In general the method of microscopic imaging of nanomaterials is described. Briefly, a sample of nanomaterials in solution was dropped onto a silicon wafer or thin glass slide and allowed to dry. For silver nanowires, the sample was sputter-coated in gold prior to imaging. Samples were then imaged using a Zeiss Ultra Plus field emission SEM. Size distribution measurements were obtained using Image J software. Statistical analysis on length measurements was carried out using the GraphPad Prism statistics package (GraphPad Software, Inc., USA).

2.2.4.4 Nanoparticle Tracking Analysis

Concentration determination of silica nanospheres was performed (courtesy: Mr. Kieran Crosbie-Staunton and Mr. Ciaran Maguire, TCD) using the NanoSight NS500 (NanoSight, Wiltshire, UK) with the on-board NTA software. Nanoparticles in sterile water were briefly sonicated and loaded into the NanoSight NS500. The dilution was adjusted so that the requisite amount of tracks (>200) could be recorded whilst not exceeding the max particle tracks per analysis frame. Camera sensitivity was adjusted and each sample was recorded six times and average particle concentration was calculated via onboard algorithms which account for liquid viscosity and ambient temperature.

2.2.5 Functionalisation of nanomaterials with antibody

2.2.5.1 Nanomaterial stock concentration determination

The stock concentration for each nanomaterial solution (particles/ml) was determined before the functionalisation process.

Flow cytometry

For non-spherical nanoparticles, a time-restricted flow cytometry approach was used to estimate particle concentrations. Firstly, the flow rate ($\mu\text{l}/\text{min}$) of the flow cytometer was quantified by running a known volume of liquid in the cytometer for a fixed time period (3 min), and measuring the volume of remaining liquid. Subtraction of remaining liquid volume from original volume, divided by elapsed time gave the average flow rate [low flow: 4 $\mu\text{l}/\text{min}$, medium flow: 42 $\mu\text{l}/\text{min}$, high flow: 56 $\mu\text{l}/\text{min}$]. Nanowires were suspended in sterile water and the dilution was adjusted so that the number of events measured in the flow cytometer did not exceed 500 events/minute at medium flow rate. Nanowire concentration was calculated by measuring the number of events over 5 min and subtracting the mean number of events in the filtered water system (background). All measurements were carried out in triplicate. Finally, the concentration was multiplied by

the dilution factor to reach the stock concentration. A sample table of calculation is included below as illustration.

Table 2.9 Sample data calculation of nanowire concentration using flow cytometric approach. This table shows the method used to calculate the concentration of nanowires. First, the flow rate was measured. Next, the sample was read in triplicate for 5 min. The average Events/ml was calculated, and the number of events occurring in a water sample alone was subtracted to give the corrected events/ml value. Finally, this value was multiplied by the dilution factor to estimate the nanowire concentration.

	Flow Rate	Time	Events	Events/ml	Corrected Events/ml	Dilution factor	NW/ml
Read 1	42 µl/min	5 min	5634	26829	26522	1000	2.7x10 ⁷
Read 2	42 µl/min	5 min	6201	29529			
Read 3	42 µl/min	5 min	5983	28490			
Water	42 µl/min	5 min	370	1761			

2.2.5.2 Surface functionalisation of nanomaterials

Nanomaterial silanisation

Nanomaterial agglomerates were sonicated (135W at 42KHZ) until dissociated as determined at 40X bright-field magnification. Nanomaterial surfaces were silanised using APTES. Briefly, a solution of (3-Aminopropyl)triethoxysilane (APTES)/ammonium hydroxide/isopropanol (10:10:1000 v/v) was added to the dissociated nanomaterial, vortexed for 10 min and allowed to stand overnight at 4°C. Nanomaterials were pelleted by centrifugation at 15,000 rcf for 15 min and washed in sterile water 3 times.

Antibody conjugation to silanised nanomaterials

1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was used to facilitate the coupling of antibody to particle surfaces through the creation of an amide bond between the carboxyl terminals of proteins and the amine-coated particle (Figure 2.2). The carboxyl groups on antibodies were activated through incubation in 500 µl EDC solution prepared in PBS pH 6.4 (0.2 g/ml). The activated antibodies (20µg antibody/1x10⁷ nanoparticles) were added to nanomaterials (in PBS pH 7.4), wrapped in tinfoil, mixed through vortex for 20 min at room temperature and placed on a rocker for 2 days at 4 °C. Alternatively, nanomaterials could be sonicated at room temperature (water bath temperature below 30°C) for 3 hours. Antibody-conjugated nanomaterials were washed

three times (pelleted by centrifugation at 15,000 rcf for 15 min) and re-suspended in sterile water (pH 7.4).

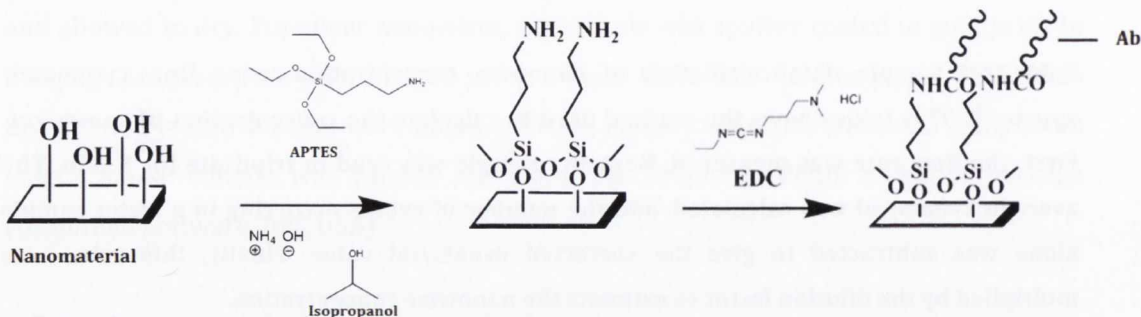


Figure 2.2 Nanomaterial silanisation and antibody conjugation process. Nanomaterials are silanised using APTES in an isopropanol solvent. Ammonium hydroxide catalyses the reaction. Following silanisation, EDC facilitates the formation of an amide bond between the amine-terminated APTES (acting as a linker) and the carboxyl terminals on the antibody. APTES; (3-Aminopropyl)triethoxysilane, EDC; 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, NHCO; Amide bond, Ab; Antibody.

2.2.5.3 Observation of EDC reaction temperature by flow cytometric approach

Observation of the effect of heat (generated by sonication) on the efficiency of EDC coupling reaction of antibody to the silanised nanowire surface was assessed by flow cytometry by measuring the %Parent of the reaction sample containing a fluorescent signal (indicating antibody binding to nanomaterial surfaces) at 30 min time-points. Briefly, silanised nanomaterials were placed in an Eppendorf and EDC and anti-CD340-AlexaFluor488 were added to the reaction (reaction volume = 1000 μ l). The reaction was placed under sonication (135 W at 42 KHZ) and the temperature was recorded. The temperature of the water bath was recorded and a 2 μ l of the sample reaction was withdrawn from the Eppendorf every 30 min. The reaction extract was immediately analysed on a flow cytometer using a SSC-H vs FITC-H bi-exponential plot. The %Parent of the total nanowire population, containing a fluorescent signal was recorded. When the %Parent value started to recede, additional EDC was added to the reaction mixture. 30 min later, ice was added to the water bath to cool the reaction and recordings were continued to 240 min.

2.2.6 Characterisation of antibody-conjugated nanomaterials

2.2.6.1 Fluorescent confocal microscopy

Functionalised nanomaterials were aliquoted on to a glass slide (Thermo Scientific, USA) and allowed to dry. Dried nanomaterials were mounted in fluorescent mounting medium (Dako, Denmark), sealed with a coverslip and imaged using the LSM 510 confocal microscope (Zeiss, Germany). Images were captured using LSM software and mean fluorescence intensity data was collected for each wire batch ($n = 20$) using the line profile function on the LSM software (with thanks to Dr Tatsiana Rakovich for assistance with data capture and analysis).

2.2.6.2 Atomic Force Microscopy

Nanomaterials were compared before and after functionalisation with antibody. Briefly, nanowires in solution were dropped on a glass slide and allowed to dry before topographical assessment of nanowires was conducted via dry phase AFM. This approach used the NTEGRA Spectra system (NT-MDT, Moscow, Russia) in tapping mode (performed by Dr Namrata Jain, TCD) using MLCT cantilevers (Veeco; Bruker, Billerica, MA). Images were acquired with a resolution of 512×512 pixels and a scan rate of 0.4 Hz.

2.2.6.3 Helium-ion microscopy

Nanomaterials in solution were dropped on to polished cover slips and allowed to dry overnight. Nanowires were examined under Helium ion microscopy by Dr Alan Bell (TCD) with preparation assistance from Dr Dania Movia (TCD). Images were processed using ImageJ software using the CLAHE plugin to enhance local contrast.

2.2.6.4 Flow Cytometry

Quantitative analysis of antibody-functionalised nanowires and spheres was performed using the BD FACS CantoA equipped with a 488nm laser. Briefly, samples were diluted in filtered water and sonicated for 5 seconds prior to analysis. The voltage applied to the SSC detector was adjusted so that filtered water alone obtained an event rate of between 5-10 events/second. Light scatter signals were thresholded at 200 on the FITC channel. Data was visualised in the SSC-Height vs. FITC -Area bi-axial linear dot-plot. Estimation of nanowire concentration was achieved by measuring the number of events over 3 minutes in triplicate at medium flow rate ($42 \mu\text{l}/\text{min}$) and subtracting the mean number of events in the filtered water alone (background). Finally, the concentration was multiplied by the dilution factor to reach the stock concentration.

2.2.6.5 Nanoparticle Tracking Analysis

Sizing and concentration determination of functionalised spherical particles was performed as previously described in 2.2.4.4.

2.2.6.6 Spectroscopy

Functionalised and non-functionalised nanospheres were spectroscopically examined using the Epoch spectrophotometer (BioTek Instruments, Inc., USA). Briefly, stock silica nanoparticles, stock fluorescent antibody solution, functionalised nanosphere solution and water were separately aliquoted in triplicate to a 96-well plate. An absorbance spectrum scan of all samples was made using the spectrum scan software (Gen5 v1.11, BioTek Instruments).

2.2.6.7 Gel Electrophoresis

Gel electrophoresis was used to compare silica nanospheres before and after functionalisation with antibody. Nanospheres were electrophoresed in agarose gel 0.2% (Table 2.10) in 0.5X TBE Buffer (Table 2.11) for 30min at 150V (Hanauer et al 2007).

Table 2.10 Agarose gel 0.2%

Agarose	0.1 g
Distilled water	50 ml

Table 2.11 5X TBE Buffer pH8.3

Tris-Base	27 g
Boric Acid	13.75 g
0.5M EDTA pH8	10 ml

2.2.7 Assessment of functionalised nanowire stability using flow cytometry

A flow cytometry-based approach was developed to assess the stability of functionalised nanowires over a 28-day period. This was done in order to demonstrate the suitability of the functionalised nanoprobe for long term storage, as this may be necessary in a clinical diagnostic setting. Briefly, the functionalised nanoprobe was measured on the day of functionalisation with fluorescent antibody (anti-CD340-AlexaFluor488). The quadrant gate was placed on the SSC-H vs FITC-H bi-exponential plot so that the functionalised nanoprobe (Q2) could be differentiated from the anti-CD340-AlexaFluor488 antibody alone (Q4). The %Parent falling into Q4 was assessed weekly in the functionalised nanoprobe population in order to see if any of the conjugated antibody had detached from the nanomaterial surface.

2.2.8 Initial proof-of-concept testing of developed nanoprobe platform

Following the validation of anti-ErbB2 antibody performance and antibody conjugation to the nanowire surfaces, and initial proof-of-concept test was performed to assess the potential for the nanowire system to detect a specific target protein (i.e. sErbB2) versus a non-specific target protein (i.e. Bovine Serum Albumin). Serum albumin was chosen as a comparator as it is the most abundant protein component found in blood.

2.2.8.1 Detection of sErbB2 using the developed nanowire platform

Briefly, silica nanowires functionalised with Trastuzumab (non-fluorescently tagged), were incubated in PBS containing either 1µg/ml recombinant sErbB2 (eBioscience Ltd, UK) or 1µg/ml BSA (Gibco, USA) for 2 hrs at room temperature with gentle rocking. The samples were pelleted by centrifugation (10,000 rcf for 10 min) and washed twice with PBS. The anti-CD340-AlexaFluor488 antibody (1µg/ml) was then added to each sample and incubated for 2 hrs at room temperature under tin foil to protect the fluorophore. Each sample was washed as before and an aliquot of each sample was mounted on a coverslip for subsequent confocal imaging.

The remainder of the sample was analysed by flow cytometry. Briefly, the functionalised nanowire population was plotted on a SSC-A vs FSC-A bi-exponential plot and a gate (P1) was placed on the population. The events recorded in the gate were then reflected into a FITC-A histogram in order to visualise the optical signal generated from each nanowire sample.

2.2.9 Further assay development & FACS analysis approaches

2.2.9.1 Antibody pair selection for nanoprobe assay

Antibody pair selection for the further development of the nanoprobe-based sandwich assay was carried out following validation of a range of anti-ErbB2 antibodies and extensive testing performed by Progenika Biopharma in order to determine the most efficient antibody combination for the developing nanoprobe-based assay. For the ErbB2 target, the capture antibody selected was the FITC-tagged mouse anti-ErbB2 monoclonal antibody (mAb) (BMS120, eBioscience Ltd, UK) and the complimentary detection antibody selected was the goat anti-ErbB2 polyclonal antibody (pAb) (AF1129, R&D Systems, Inc, USA). The selected detection antibody was not available as ready-made with

a fluorescent tag, so a secondary antibody (donkey anti-goat pAb) with phycoerythrin (PE) tag was used as a fluorescence reporter.

2.2.9.2 Nanoparticle assay development

The nanoparticle assays were developed from bead assay protocols provided by Progenika Biopharma. The concentration of nanoparticles used in the assay was calculated through comparison to theoretical reactive surface area of the bead assay (i.e. theoretical surface area of bead * number of beads used per assay sample). Various concentrations of capture antibody-nanoparticles, detection antibody and fluorescent secondary antibody were trialled during the optimisation process in order to obtain the best signal-to-noise ratio for assay analysis. In the case of nanowires, due to batch-to-batch variability, assay optimisation was necessary for each new batch of functionalised nanomaterials. Using reconstituted plasma allowed the variability between clinical samples to be reduced. Spiking the plasma with known concentrations of sErbB2 allowed testing of the detection limits of the system.

2.2.9.3 FACS analysis of nanomaterial-based assays

All FACS analysis for all the nanomaterial-based assays was carried out on the BD FACS Canto A and followed the same essential steps. Firstly, controls (filtered water, non-fluorescent nanomaterial profiles, purified fluorescent antibodies, antibody-conjugated nanomaterials) were used to define populations of interest and to rule out fluorescence contamination from assay components other than those intended. The positive fluorescent threshold signal and compensation setup was done using purified fluorophore and functionalised wire fluorescent signature and background signal levels were identified from non-fluorescent material. Following sample capture, samples were gated on control populations (antibody-conjugated nanomaterials) and comparison of detection antibody fluorescent signals were compared between samples and expressed as median fluorescent intensity, %parent or ratio of fluorescent-to-non-fluorescent signal.

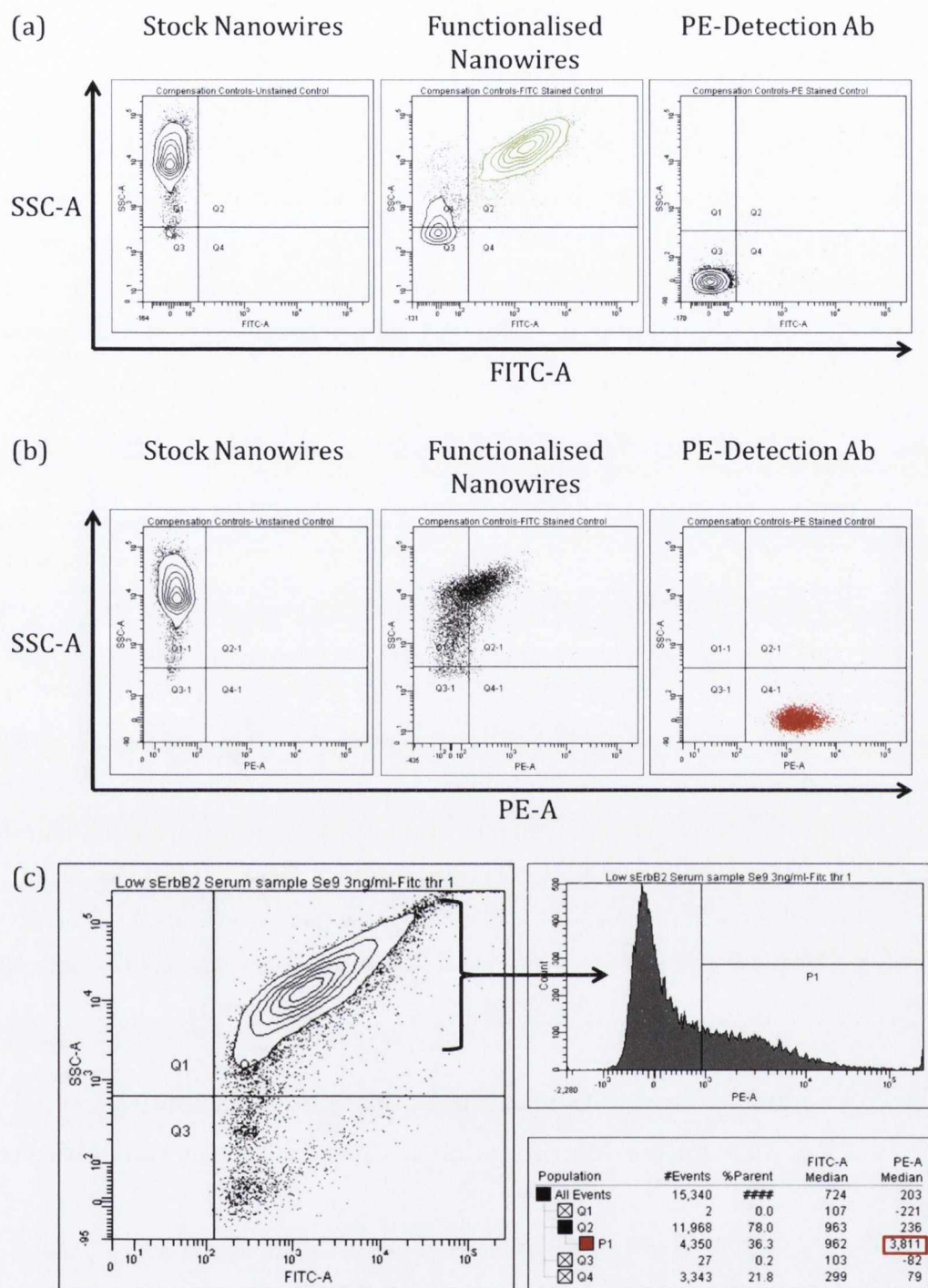


Figure 2.3 FACS analysis strategy for nanomaterial-based assays. Controls (non-fluorescent stock nanomaterial profiles, antibody-conjugated nanomaterials, purified fluorescent antibodies (Left to Right in (a) and (b)) were used to define populations of interest (placing the quadrant to isolate all relevant populations) and to compensate for fluorescence contamination from assay components other than those intended. Compensation setup was done and samples were acquired and visualised in the SSC-A vs FITC-A bi-exponential plot (c). Following sample capture, the events occurring in Q2 (antibody-conjugated nanomaterials) were reflected into a PE-A histogram and comparison of detection antibody fluorescent signals were assessed as median fluorescent intensity (PE-A Median).

2.2.9.4 Detection of sErbB2 in spiked plasma

In order to assess the detection limits of the nanoprobe systems in relevant clinical samples, the functionalised nanosphere and nanowire assays were tested in reconstituted blood plasma containing spiked concentrations of sErbB2 (range: 100 ng/ml to 0 ng/ml). In this experimental case, the nanomaterials were functionalised with a fluorescent (FITC) anti-ErbB2 antibody (BMS120-FI, eBioscience Ltd, UK) to allow better nanoprobe visualisation in the flow cytometric system. Following incubation of the nanoprobe with the blood plasma, the detection antibody and PE-labelled (Phycoerythrin) fluorescent secondary antibody were applied.

2.2.9.5 Comparison of fluorophores

A comparison of fluorophores was conducted to identify a suitable replacement for FITC fluorophore. Three fluorophore-linked antibodies were compared based on their similar spectral emission wavelengths (i.e. ~520nm), FITC-antibody (eBioscience, USA), AlexaFluor 488-antibody (Biolegend, USA) and CF488-antibody (Sigma-Aldrich, USA). Each antibody was EDC-conjugated to glass substrate or silica nanowires based on antibody concentration and the samples were examined by fluorescence-based microscopy (glass and nanowires) and flow cytometry (nanowires) with FITC threshold applied. For microscopic analysis of glass, the mean fluorescent intensity of each image was calculated from 20 randomly selected areas using ImageJ software. For microscopic analysis of wires, the mean fluorescence intensity was measured along the length of the wire using line profile function on the LSM software (Zeiss, Germany).

2.2.10 Analysis of nanowire signatures using flow cytometry

2.2.10.1 Size distribution determination of silver nanowires and polystyrene spheres

Four batches of silver nanowires (median lengths by SEM: 26 μm , 21 μm , 6 μm & 2 μm) [AgNWs] (Seashell Technologies, USA) and six batches of polystyrene spheres (15 μm , 10 μm , 6 μm , 4 μm , 2 μm , 1 μm) (F-13838, Invitrogen) were used to examine uniaxial nanowire signatures in flow cytometry. The size distribution of each batch was characterised using scanning electron microscopy (SEM). Briefly, a sample of AgNWs or spheres in solution were dropped onto a silicon wafer and allowed to dry. Each AgNW sample was sputter-coated in gold prior to imaging. All samples were then imaged using SEM. Size distribution measurements were obtained using Image J software. At least 100 length measurements were taken for each batch of AgNWs and statistical analysis was carried out using the GraphPad Prism software (GraphPad Software, Inc., USA).

2.2.10.2 Data capture

Light scatter properties of AgNWs and polystyrene spheres were observed using the BD FACS CantoA (BD Biosciences, USA) flow cytometry system equipped with a 488nm laser with the accompanying FACS Diva Software. Briefly, all samples were diluted in filtered water so that the event rate was maintained at ~200 events/sec. The voltage applied to the SSC detector was adjusted so that filtered water alone obtained an event rate of between 5-10 events/second. Light scatter signals were thresholded at 200 on the SSC channel. All samples were measured at a low flow rate, in triplicate, using identical cytometer settings. The voltage applied to SSC and FSC detectors was adjusted so that data from all the samples would fall within the linear plots.

2.2.10.3 Comparison of scatter properties of AgNWs with spherical particles using flow cytometry

A comparison was made using the Forward Scatter (FSC) and Side Scatter (SSC) parameters [Area (A), Height (H) & Width (W)]. Triplicate readings were made for each sample (100,000 events recorded for each sample) and the median values for each parameter were plotted and analysed using GraphPad Prism software (GraphPad Software, Inc., USA). Additional visualisation of the data obtained for SSC-W measurements were performed using FlowJo V10 software (FlowJo LLC, USA).

2.2.10.4 Analysis of AgNW length-associated response using SSC-W gating strategy

A novel gating strategy was developed to analyse the length-associated side scatter response in AgNW populations. Data for each nanowire sample was viewed on the SSC-Width (SSC-W) histogram plot and regular gatings (P1-P25) were applied to cover the entire response range of the SSC-W histogram. Data from each gate was projected into a SSC-Height (SSC-H) vs. SSC-Area (SSC-A) bi-linear plot and visualised using FlowJo V10 software (FlowJo LLC, USA). The percentage population falling within each gate (P1-P25) was calculated by FACS Diva Software (BD Biosciences, USA) and rounded to the nearest percentage for ease of analysis.

2.2.10.5 Curve fitting calculation for AgNW optical response.

For AgNW and polystyrene sphere samples, SSC-H and SSC-A values for each particle event were exported and plotted on an X-Y scatter plot using GraphPad Prism software (GraphPad Software, Inc., USA). The on-board linear regression model was applied to each dataset and the slope, inverse slope and R^2 value were calculated.

2.2.11 Development of novel technological platform for fluorescent nanoparticle assays

2.2.11.1 Setup of integrated microfluidics and optical system for nanoparticle analysis

A custom-made microfluidics liquid handling platform coupled to a custom-made focused flow cell (Cellix, Ireland) and a fluorescent detection system (Radisens Diagnostics, Ireland) were integrated to form a technological platform (RDS-CLX) capable of measuring fluorescently-tagged nanoparticles. The below figure (Figure 2.4) describes the setup of the system. The Mirus ExiGo microfluidic pump (Figure 2.4, 1) allowed the rate of nanoparticle solution dispensation to be tightly regulated. In addition to the central sample inlet on the focused flow cell (Figure 2.4, 2), two other inlet ports facilitated the focusing of the sample stream across the illumination point (Figure 2.4, 3). Emitted fluorescence from the sample was captured in the same objective lens and signal was sent to the fluorescence detector for digitization (Figure 2.4, 4), with subsequent analysis carried out by custom-made software (Radisens Diagnostics, Ireland).

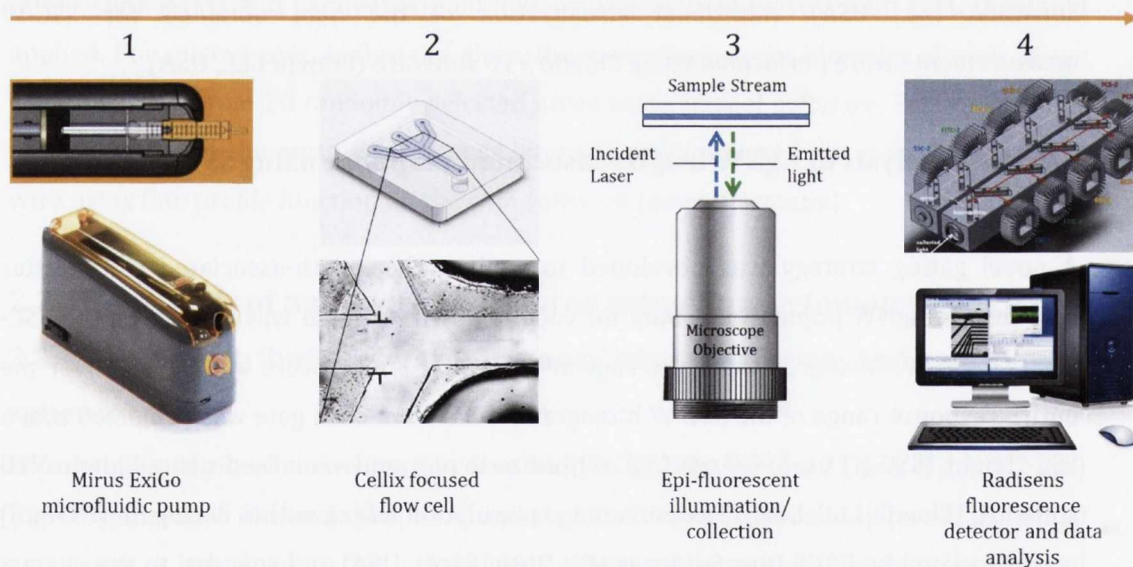


Figure 2.4 Setup of integrated microfluidics and optical platforms for the analysis of fluorescently-tagged nanoparticles. The orange arrow indicates the series linkage (1-4). The Mirus ExiGo microfluidic pump (1) (schematic above, photo below) was used to control the flow rate of fluorescent nanoparticles in solution through the system. The integrated fluidics on the custom-made flow cell (2) (schematic above, magnified view below) allowed the separation and focusing of nanoparticles in the sample stream across the incident laser

light (3). The fluorescent light emitted from the nanoparticles was collected via the objective and captured by the fluorescence detector (4) for subsequent data analysis.

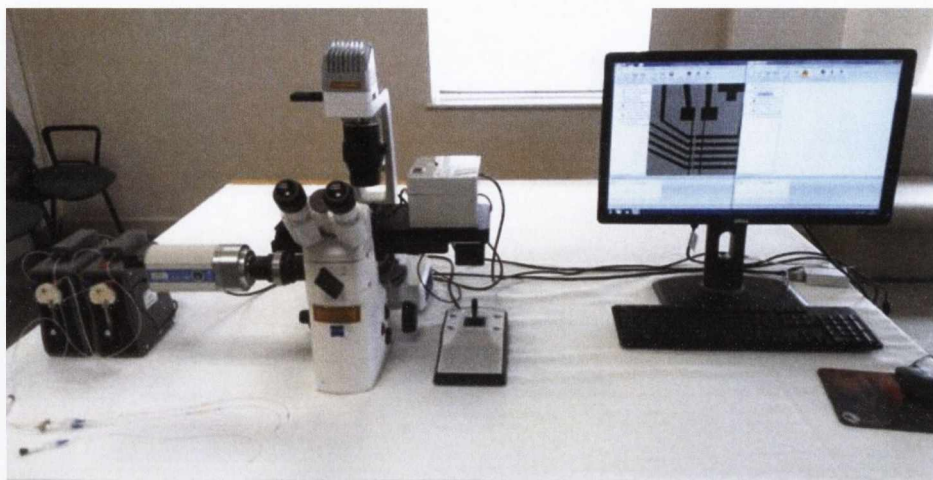


Figure 2.5 Example benchtop setup of microfluidics and optical platform for nanoparticle analysis. In this instance, the custom fluorescent detector is replaced by a CCD camera.

2.2.11.2 Feasibility testing of integrated technological platform for nanoparticle analysis

Preliminary testing was carried out on the integrated technological platform to assess the potential for its use in capturing light scatter signals and measuring fluorescent signatures from nanomaterials conjugated with fluorescent antibodies. The integrated platform consisted of microfluidics, optical acquisition unit and computer software components.

2.2.11.3 Comparison of particle detection in RDS-CLX acquisition system vs conventional flow cytometer.

Firstly, a range of spheres of different diameters (100, 200, 280, 400, 600 & 3200 nm) were measured in the RDS-CLX system and a conventional flow cytometer (FACS CantoA), by their SSC properties. Briefly, the sheath fluidics were primed and the capture optics were aligned with the focused flow cell on the microscope stage with the aid of calibration beads. The scatter signal from the beads could be viewed on an auxiliary oscilloscope and the laser orientation was tuned until the maximum signal readout was visible on the oscilloscope. The syringe containing the nanoparticle solution was then loaded in the pump and the sample input rate was adjusted so that the frequency of the signal peaks could be discriminated. Once the sample input rate was optimised the trigger threshold

value was set on the acquisition software and the data was acquired. Particle acquisition was set to be triggered on side scatter height (SSC-H) parameter and 2000 events were captured for each sample. Both sets of data were exported for analysis using custom software developed for the new platform.

2.2.11.4 Detection of fluorescent nanoparticles using the RDS-CLX system

The RDS-CLX system was used to detect FITC-tagged florescent beads (3.2 μm & 7.4 μm) and anti-CD340-AlexaFluor488 functionalised nanowires (sincere thanks to Francesco Dicorato (Cellix) for assistance with the alignment and acquisition). The optics were aligned with the focused flow cell on the microscope stage as before. Particle acquisition was set to be triggered on FITC parameter and 10,000 events were captured for each sample. Both sets of data were exported for analysis using custom software developed for the new platform.

2.3 Methods for Chapter 4

2.3.1 Cell culture

Two cell lines were used in detection of PSA experiments. Cell lines were chosen from the American Type Culture Collection (ATCC) biological resource centre catalogue (VA, USA) and were selected to reflect a range of target protein expression levels. All cell lines were maintained between passage 3 and 20 in basal media containing 1% Penicillin/Streptomycin (Gibco) supplemented with Fetal Bovine Serum (FBS, South American origin, Gibco) (Table 1).

Table 2.12 Cancer-derived cell lines for use in *in vitro* nanowire assays.

Cell line	Basal Medium†	Serum Supplement*	Cancer type
LnCap	RPMI	10% FBS, 1% P/S	Prostate Carcinoma
PC3	F-12K		Adenocarcinoma
* FBS: Fetal Bovine Serum; P/S: Penicillin/Streptomycin; †RPMI: Roswell Park Memorial Institute Medium.			

2.3.2 Antibody pair selection for EGFR and PSA biomarker detection

For the EGFR target, the capture and detection antibody selected was the goat anti-EGFR pAb (AF231, R&D Systems, Inc, USA) For the PSA target, the capture antibody selected

mouse anti-PSA mAb (MAB1344, R&D Systems, Inc, USA) and detection antibody was mouse anti-PSA mAb [A67-B/E3] (Abcam plc, Cambridge, MA). Each capture antibody was fluorescently tagged with CF488 and each detection antibody was tagged with CF633.

2.3.3 Nanowire functionalisation

CoNi nanowires were used for experimentation and the nanomaterials were functionalised with antibody as before, silanisation with APTES followed by EDC-driven conjugation of capture antibody to the surface. Prior to use, the wires were validated by flow cytometric approaches as previously described.

2.3.4 ELISA quantification of soluble proteins in biological samples

2.3.4.1 Ethical approval for study

Ethical approval permitting the receipt, storage and handling of human blood samples to facilitate the development and testing of a novel diagnostic nanoprobe was gained (See Appendix 1). Blood samples were obtained from Progenika Biopharma (Spain) and were selected to reflect a range of disease stages.

2.3.4.2 Soluble protein quantification in clinically relevant biological samples and cell lysates

Soluble proteins ErbB2 and PSA were measured in clinically relevant biological samples and cell lysates using ELISAs according to manufacturer's instructions. Quantification of soluble protein concentration was carried out in triplicate for each sample.

2.3.5 Detection of cancer biomarkers using the developed nanowire platform

Detection of cancer biomarkers using the nanowire assay was carried out with assistance from Dr Tatsiana Rakovich and followed the same protocol developed for the detection of sErbB2 in the single analyte system and incorporated the improvements and modifications (e.g. preferred fluorophore selection were CF488 for the capture antibody) to the initial testing system.

2.3.6 Comparison of theoretical reactive surface area of nanowire and ELISA platforms

In order to highlight one of the potential advantages of using a nanowire-based detection system over the conventional ELISA systems, a comparison of the theoretical reactive surface area of each approach was estimated. The theoretical reactive surface was defined as the surface area on which a capture antibody could be immobilised (ie. an ELISA plate well or a defined number of nanowires), and thus the area on which an assay of a molecular target could take place. For the ELISA well, the average area of the floor of a flat-bottomed well was used (based on well diameter of 6.4 mm) and calculations ignored the well wall area. For the nanowire platform, the theoretical reactive surface area was calculated by multiplying the theoretical surface area of one nanowire (assuming diameter of 150 nm and length of 10 μm) by the number of nanowires necessary per assay sample (ie. 100,000 nanowires).

2.4 Synopsis of techniques used to examine nanomaterials

Below is a synopsis of the different techniques used to examine NMs during the work carried out in this thesis. The table describes the techniques associated with examining single members of a NM population as well as those which are used to assess NMs on a population/bulk basis. Some of the advantages and disadvantages of each technique in the examination of NMs are noted.

Table 2.13. Synopsis of techniques used to examine nanomaterials

	Technique	Advantages	Disadvantages
Analysis of single NM entities within a NM population	Fluorescent Confocal Microscopy	- Allows visualisation of fluorescently tagged antibodies on NMs - Relatively rapid compared to AFM	- Destructive: Sample is inseparable from mounting media following analysis - Reliant on fluorophore selection and setup
	Atomic Force Microscopy (AFM)	- Useful for examining surface topology - Non-destructive preparation - Simple preparation method	- Heavily dependent on user expertise - Labour intensive data acquisition and analysis
	Helium-Ion Microscopy	- High resolution imaging - Simpler preparation	Not necessary for general bulk NM assessment

	(HIM)	method vs SEM	• Heavily dependent on user expertise • Expensive • Sample may be damaged during
	Scanning electron microscopy (SEM)	• High resolution imaging • Samples require sputter coating prior to imaging	• Not necessary for general bulk NM assessment • Heavily dependent on user expertise • Difficult to visualise proteins • Sample is altered during analysis
	Light Microscopy	• Non-destructive • Simple preparation method • Inexpensive	• More difficult to see low-contrast NMs (e.g. non-metallic NMs such as silica NMs) • Lower resolution imaging vs SEM and HIM
Analysis of NM populations	Flow cytometry	• High throughput: Can assess sample populations >1,000,000 events • Allows visualisation of fluorescently tagged antibodies on NMs • Multi-parametric data collected for analysis	• Heavily dependent on user expertise • Sample is consumed/destroyed during analysis • Not suitable for NM which absorb fluorescent light (e.g. Ag NWs)
	Nanoparticle tracking analysis (NTA)	• High throughput: Can assess very large sample populations (>1,000,000 events) • Good for spherical NMs • Inbuilt concentration calculation	• Particle tracking algorithms not suitable for assessing non-spherical NMs such as NWs • Highly susceptible to user error and environmental conditions • Time consuming
	Absorbance Spectroscopy	• Simple sample preparation and fast, automated data acquisition time • Quick and rough	• Mono-parametric data output • NW geometry is not ideal for absorbance studies • Dispersion of incident light

		assessment of antibody coupling success	by NMs in solution must be taken into account when assessing absorption readings
	Gel Electrophoresis	• Simple sample preparation • Fast • Can provide data on particle surface charge • Suitable for particles <100nm diameter	• Not suitable for NWs. NWs become trapped in the gel matrix • No reliable quantitative data obtainable from this technique • Sample is consumed/destroyed during analysis

2.5 References

Bunn, P., et al. (2001) Expression of her-2/neu in human lung cancer cell lines by immunohistochemistry and fluorescence in situ hybridization and its relationship to in vitro cytotoxicity by trastuzumab and chemotherapeutic agents. *Clinical Cancer Research* **7**: 3239-3250.

Subik, K., et al. (2010) The expression patterns of er, pr, her2, ck5/6, egfr, ki-67 and ar by immunohistochemical analysis in breast cancer cell lines. *Breast Cancer: Basic and Clinical Research* **4**: 35-41.

Chapter 3

Results: Part I

Development of nanoprobe for detection of the sErbB2 cancer biomarker

3.1 Introduction

3.1.1 Nanomaterials

A nanomaterial can be defined as any material where at least one of its dimensions can be measured on the nanometre (nm) scale, and are usually referred to as such when the properties of the nanoscale features contribute to the functional properties of material. The definition of a nanomaterial adopted by the EU commission (2011) states the following;

“A natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm - 100 nm.

In specific cases and where warranted by concerns for the environment, health, safety or competitiveness the number size distribution threshold of 50 % may be replaced by a threshold between 1 and 50 %.”

The classification of nanoscale materials is based on the number of dimensions not on the nanoscale (<100nm). An in-depth overview of the categorisation of 0-, 1-, 2- and 3-dimensional nanomaterials (Illustrated in Table 3.1) is provided in (Ashby et al. 2009).

Table 3.1 Categorisation of nanomaterials. Nanomaterials can be classified into one of four groups, based on their sub-100nm dimensionality. This table synthesizes the criteria for assigning their dimensionality, examples of the nanomaterials in each category and includes an illustrative example of each material. Table adapted from (Ashby et al. 2009).

Category	0-Dimensional	1-Dimensional	2-Dimensional	3-Dimensional
Dimensionality 	All dimensions ≤100nm	One dimension >100nm, two dimensions ≤100nm.	One dimension ≤100nm, two dimensions >100nm.	No bulk dimensions at ≤100nm, but composed of materials of ≤100nm.
Examples	Quantum dots, Nanospheres, Nanodiamonds, Nanostars, Liposomes	Nanowires, Nanotubes, Nanofibres	Nanofilms, Nanocoatings	Nanocrystalline & Nanocomposite materials
Illustration				

Research into nanomaterials in the field of biomedical diagnostics is conducted for a number of reasons and is driven by increasing global health demands. Primarily, materials of this size possess inherent physico-chemical properties (Roduner 2006) not found in the larger bulk materials from which they are derived, nor in their constituent particles alone. Nanomaterials exhibit high surface-to-volume ratio, high aspect ratio, controllable shape (e.g. isotropic vs anisotropic), enhanced plasmonic, electro-conductive and catalytic properties, which are amongst the main properties of nanomaterials being exploited towards their development and incorporation into the next generation of diagnostic platforms (see Chapter One). Additionally, the technologies needed to characterise nanomaterials have developed to an adequate extent facilitating nanomaterial research, development and integration into diagnostic devices.

3.1.2 Inherent properties of nanomaterials: Focus on S/V characteristics

High surface-to-volume (S/V) ratio is an inherent property of nanomaterials, with the S/V ratio increases dramatically as dimensions reach the sub-10 nanometer range (Figure 3.1). Interestingly, the magnitude of S/V ratio of nanowires is an order of magnitude larger than nanospheres of similar radius.

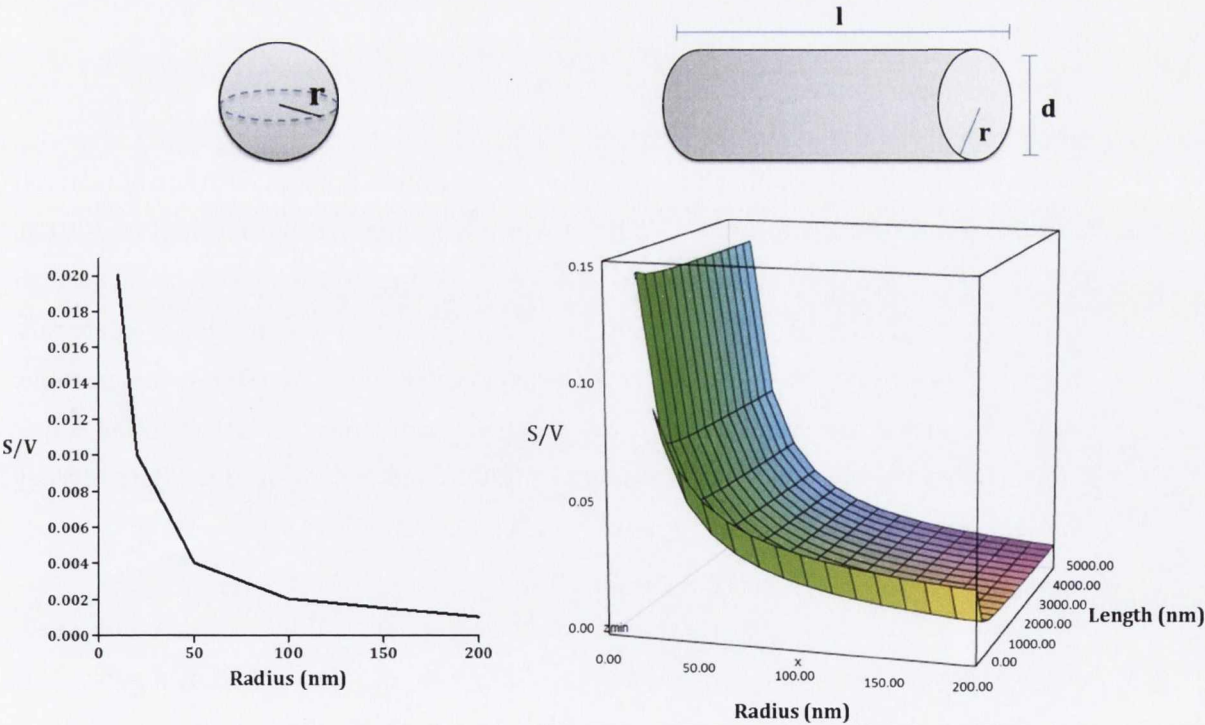


Figure 3.1 Theoretical surface-to-volume ratios for isotropic, spherical (left graph and illustration) and anisotropic, cylindrical (right graph and illustration) shape nanomaterials. A high surface-to-volume (S/V) ratio is observed in both types of nanomaterial shape as the theoretical radius of the particle tends toward 1 nm. As the radii of both nanomaterial

shapes reach 1 nm, the S/V ratio of nanowires an order of magnitude larger than that of the spheres. l: length; d:diameter; r:radius. Graphs made using GraphPad Prism for sphere graph and 3D function grapher for wire graph (livephysics.com).

This aspect ratio-related advantage of 1-D nanomaterials can be utilised to achieve the next generation of diagnostic platforms (Matlock-Colangelo & Baeumner 2014). Synthetic approaches to 1-dimension nanomaterials from metal alloys (Rheem et al. 2007) (e.g. CoNi/Au nanowires composing wires using alternating magnetic and non-magnetic materials) could increase their application into multimodal biosensing, thus offering an opportunity for assay simplification (see section 3.1.4 on Application of Nanowires for Biosensing).

3.1.3 Synthesis of nanomaterials

The factors influencing the selection of a particular synthetic route for nanomaterial production are usually dictated by the desired end use and include the associated costs of production, access to technical expertise, reliability of the synthetic route and the potential for manufacturing upscale. The synthesis of nanomaterials can be categorised into two approaches; bottom-up synthesis and top-down synthesis. Some techniques, such as nanosphere lithography (Colson et al. 2013) combine both bottom-up and top-down processes.

3.1.3.1 Bottom-up synthesis of nanowires

There is a wide variety of physical and chemical deposition approaches to nanomaterial synthesis via the bottom-up route, some of which are reviewed by Devan et al. (2012). The bottom-up approach to nanomaterial synthesis refers to their production through directed combination of substituent atoms. In the case of 1-dimensional materials, metallic nanowire synthetic routes are well investigated and understood. For example, there are several routes to achieve the synthesis and anisotropic control of silver nanowires are reviewed in Gerard and Gun'ko (2013) and include seed-mediated (Wiley et al. 2005) and template-mediated (Choi et al. 2003) synthesis.

The polyol method of silver nanowires synthesis (Fievet et al. 1989) is a common, seed-mediated approach which involves the reduction of a silver salt (AgNO_3) by a polyol (e.g. glycerol, ethylene glycol) at an elevated temperature in the presence of a stabiliser/capping agent such as cetyl trimethylammonium bromide (CTAB) (Murphy et al. 2006) or polyvinylpyrrolidone (PVP) (Sun et al. 2003). This method first involves the preferential nucleation of the decahedral multiple-twinned silver seed-particles through

Ostwald ripening, a process which is dependent on parameters such as the concentration of AgNO_3 and the molar ratio between PVP and AgNO_3 (Wiley et al. 2005). The stabilizer also acts to prevent agglomeration of the nucleating particles. Following seed formation by the initial reduction process, the polyol-assisted reduction of metal ions to the surface of the seed-particles is facilitated by the process of further heating (Wiley et al. 2005) and (Guo et al. 2009) or irradiation (termed photo-chemical synthesis) (Zhang et al. 2011). Anisotropic wire growth becomes energetically favourable once the seed adopts a multiply twinned structure (Sun et al. 2003), and this uniaxial elongation is facilitated by PVP and CTAB (Wiley et al. 2005).

Since its initial demonstration, further understanding and refinement of polyol-mediated nanowire formation (e.g. reaction temperature, reducing agent, water/glycerol ratio and the use of metal halide control agents) has led to the rapid production of nanowires with increasing scalability and dimensional control (Yang et al. 2014). Recently, further variations on the polyol-mediated route have yielded one-step synthetic approaches to directed nanowire growth with high reproducibility (Tang et al. 2014).

Template-mediated synthesis of metallic nanowires is a robustly controllable process yielding highly uniform nanowire populations. Hard template-mediated synthesis consists of depositing metals inside a pre-formed membrane template (such as porous alumina) on a sputtered surface through galvanostatic deposition (anodization). Following anodization, the synthesised nanowires are etched from the membrane by an acid bath (e.g. nitric acid), followed by dissolution of the surrounding alumina membrane by hydrofluoric acid solution (Liu et al. 2014). In this approach, the length of the resulting wires is proportional to the charge density exerted during the anodization process. Although the so-called soft-template approach is more commonly used for nanosphere synthesis (see following section), it can be used for nanowire synthesis also. For example, rod-shaped micelles composed of CTAB (Jana et al. 2001) or liquid crystalline phases (Huang et al. 2002) can be used to produce nanowire structures.

Electron-beam-induced deposition is a less-commonly used bottom-up approach which can yield nanowire structures with sub 10nm dimensions (Kramer et al. 1995, Silvis-Cividjian et al. 2003), however the necessity for an electron beam in order to facilitate this process means it is less cost effective than the other bottom-up approaches. At the moment, although template-mediated synthesis offers greater reproducibility in terms of dimensional control, seed-mediated approaches offer the better opportunity to up-scale the production of nanowires for commercial use. The advantages of using one synthetic

approach over another should be negated in coming years as our knowledge and understanding of nanowire growth mechanisms continues.

3.1.3.2 Bottom-up synthesis of nanospheres

There is a wide variety of approaches to synthesising spherical nanoparticles (Khodashenas & Ghorbani 2014, Kulkarni & Muddapur 2014, Zhang et al. 2014) with many nanospheres being developed for nanomedical applications (Tang & Cheng 2013). For synthesis of hollow shell nanospheres for example, one common route is through a surfactant-templating sol-gel approach. CTAB or PVP (Alam et al. 2014) is mixed with an oil/water (Wu et al. 2014) or ethanol/water (Chen et al. 2014) emulsion to create a surfactant template. Silicon dioxide precursors such as Tetraethoxysilane (TEOS) and silane coupling agents such as ODTES (octadecyltriethoxysilane) are then added and condense under stirring and heat to form the outer shell. Once the silica shell is formed, the CTAB template is removed by calcination at high temperatures ($\sim 500^\circ\text{C}$) (Tagaya et al. 2014) to provide the silica nanosphere. The surfactant-templating sol-gel approach has been further adapted to produce mesoporous nanospheres for multidrug delivery applications (Fang et al. 2014).

3.1.3.3 Top-down synthesis of nanomaterials

The top-down approach to nanomaterial synthesis relies on production of nanomaterials through controlled removal of material from a bulk starting material through a process such as etching (Ghafarinazari & Mozafari 2014) and exfoliation (Tour 2013). The top-down approach can produce varied nanowire structures from singular nanowire bridges to highly uniform nanomaterial arrays.

Optical lithography, a subtractive top-down approach to nanomaterial production, is considered the industry standard for lithographic fabrication of microstructures. The minimum critical dimensions achievable by lithographic approaches are proportional to the incident radiation wavelength and inversely proportional to the refractive index of the lens immersion medium. Current approaches in optical lithography favour a short wavelength (e.g. 193nm ArF laser) radiation to achieve high lithographic resolution. Interference lithography (exploiting the interference pattern produced by coincident coherent beams) and immersion lithography (employing a projection lens immersed in a high refractive index medium) result in further improvements to the minimum critical dimensions of lithographic resolution. The next generation of lithography for nanomaterial production include electron beam, nano-imprint, extreme ultra violet, X-ray,

scanning probe and electron beam induced deposition lithography approaches (reviewed in (Hobbs et al. 2012)).

Nanowires can be fabricated from layered substrates such as silicon-on-insulator (SOI), or wafers prepared by chemical vapour deposition. Ning and colleagues (2011) have produced highly uniform multi-segmented InGaAsP/InP nanowire arrays using a plasma reactive ion etching process. The resulting highly uniform nanowires were suitable for optical applications due to their narrow photoluminescence linewidth. Single suspended Au-nanowire bridge structures for biomolecular detection applications have been fabricated using electron beam lithography (Zou et al. 2009). More recently, Georgiev et al. (2014) have fabricated silicon nanowire-based field effect transistors for biosensing applications from SOI wafers. The process used a combination of electron beam lithography, reactive ion etching, photolithography, and wet etching techniques to achieve the integrated structures.

Nano-milling (nicely illustrated in (Radzuan et al. 2010)) is another top-down approach to nanomaterial fabrication (Stenger et al. 2005) and has been used to produce nanocrystal drug formulations with improved aqueous solubility (Abdelbary et al. 2014) in order to enhance the bioavailability of poorly water soluble drugs (Niwa & Danjo 2013).

3.1.3.4 Nanomaterial functionalisation: exploiting the inherent advantages of nanomaterials

In the field of biomedical diagnostics, the advent of chemical linkers has allowed the inherent properties of nanomaterials to be integrated with the intrinsic biological function (i.e. specific molecular recognition capabilities) of biomolecules such as antibodies, DNA, RNA and polysaccharides. This marriage between material engineering and biology has resulted in the expansion of new avenues exploring the function and application of nanomaterials for enhanced biological assay systems. The use of chemical linkers has three main functions: Firstly, as a barrier layer used to protect the nanomaterial from degradation (e.g. through oxidation) or to maintain the stability of a nanoparticle in solution (e.g. aqueous vs organic solution). Secondly, the linker is used as a method of coupling the biomolecule to the surface of the nanomaterial, thus adding further functional capacity to the material. Lastly, the linker can act as a spacer to separate potential fluorophores and a nanomaterial which could absorb the fluorescence (thus reducing the fluorophore emission available for subsequent signal analysis).

The choice of chemical linker (such as polyethylene glycol (PEG), (3-aminopropyl) triethoxysilane (APTES) and dextran) is usually dictated by the nanomaterial surface composition, the desired stability of the nanomaterial in a given solution, and the target biomolecule to be conjugated to the nanomaterial surface. EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) is commonly used to facilitate the coupling of antibody to particle surfaces through the creation of a stable amide bond between the carboxyl terminals of proteins and the amine-coated particle. The use of chemical linkers has enabled functionalisation of NPs with molecules such as antibodies, ligands, biocompatibility agents and drug candidates (Bhowmick et al. 2012, Costas & Olga 2013, Martin et al. 2009).

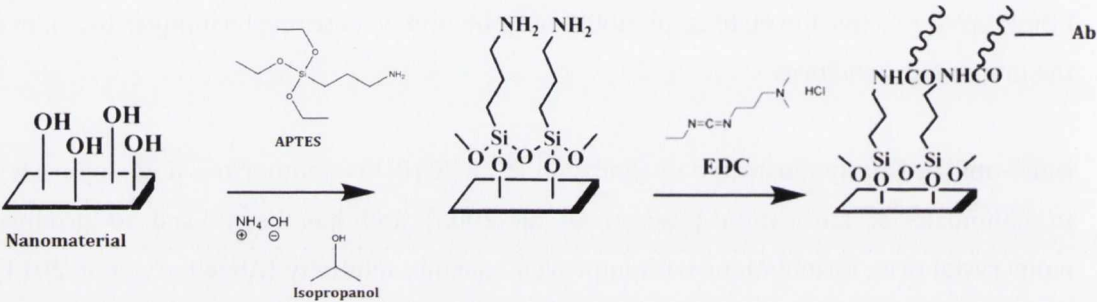


Figure 3.2 Nanomaterial silanisation and antibody conjugation process. Nanomaterials are silanised using APTES in an isopropanol solvent. Ammonium hydroxide catalyses the reaction. Following silanisation, EDC facilitates the formation of an amide bond between the amine-terminated APTES (acting as a linker) and the carboxyl terminals on the antibody. Mechanism adapted from (Thakurta & Subramanian 2012).

3.1.4 Applications of Nanowires for Biosensing

Biosensors can be generally classified into optical, electrical/electrochemical, mechanical and magnetic biosensors. Nanomaterials have been developed and applied to each of these sensor types (Jariwala et al. 2013, O'Mahony & Wang 2013, Suresh & Arivuoli 2012, Tamayo et al. 2013). In each instance, capitalising on the advantages of nanomaterial use requires the simultaneous development of a complementary technological platform. Several nanowire applications to biosensing are highlighted here, as well as the complementary technological approach necessary to assessing these nanomaterial-based assays.

3.1.4.1 Nanowires in optical biosensors

In terms of optical sensors, single-walled carbon nanotubes (SWCNTs) have been applied to several fluorescence-based biosensing applications (Kruss et al. 2013). Optical signal transduction is based on quenching/recovery of near infrared (NIR) fluorescence emission of SWCNTs upon analyte binding (reviewed in (Li & Shi 2014)). Strano and colleagues have developed several SWCNT-based sensors for the detection of glucose (Barone et al. 2005, Barone & Strano 2006), nitric oxide (Kim et al. 2009), adenosine triphosphate (Kim et al. 2010) & hydrogen peroxide (Heller et al. 2008). Interestingly, the reversible response due to competitive binding (quenching/recovery following dissolution/binding of analytes from the nanoprobe) of these particular SWCNT assays allows the potential for continuous monitoring of analytes in certain sensing applications. For the SWCNT assays mentioned above, customised technology used a photodiode laser (Excitation at 785 nm) as illumination source and signal capture via a near infrared fluorescence microscope coupled to an Indium Gallium Arsenide (InGaAs) array detector (measures optical power in the NIR range) and a spectrograph (separates light into a frequency spectrum and records the signal). In a separate approach, the advantageous Raman scattering properties of SWCNTs have resulted in their exploitation towards biosensing assays. The use of SWCNTs containing different carbon isotopes (i.e. C12 and C13) allowed the shifted Raman features of both isotopes to be used for multiplexing of analytes with analysis carried out using a unmodified micro-Raman instrument (Chen et al. 2008). Optically encoded nanowires constructed from alternating metal sections, such as those produced by Tok et al. (2006) have provided the potential for the use of nanowires in multiplexed biological assays. Indeed, in this instance, the developed technology platform was used to detect three biological analytes simultaneously. Detection required the collection of multiple image pairs (brightfield/fluorescence) using microscopy, followed by analysis. In addition to optically encoded nanowires,

magnetically encoded nanowires are also being developed for multiplexed biosensors (see section on Nanowires in magnetic biosensors).

3.1.4.2 Nanowires in electrical/electrochemical biosensors

Nanowire integration into electrochemical sensors has been extensively demonstrated. In the nanowire-based electrochemical biosensor developed by Fahmy and colleagues (2010), function of the system relies on the operation of a microfluidic liquid handling system to; move the biological sample (whole blood) across the biochip surface (allowing interaction between the capture antibodies and the target analyte), wash the biochip following binding of the analyte, and transport the released antibody-analyte complex to a separate chamber for detection by the complementary antibody-conjugated nanowire sensor. Signal detection in this system requires assessment of the electro-conductive potential of the nanowire. Chang et al. (2011) have developed a similar nanowire biosensor for cancer biomarker quantification in whole blood, integrating a liquid handling system and polycarbonate filter (allowing removal of red blood cells from whole blood) upstream of the electro-conductive nanowire assay. This system setup used 500 μ l whole blood (more than 10 times the volume needed by ELISA) to run the assay. This may have repercussions in instances where sample volumes are limited. Recently, a resistance-based silicon nanowire detection platform has been developed to detect the prostate cancer biomarker 8-hydroxydeoxyguanosine in a single analyte system (Mohd Azmi et al. 2014). Though extensively researched and highly sensitive, it should be borne in mind that nanowire-based electro-conductive systems still have their limitations. For one, such systems are particularly sensitive to changes in ionic strength of the buffer solution and susceptible to non-specific binding limitations.

3.1.4.3 Nanowires in mechanical biosensors

Several nanomechanical biosensors incorporate nanowires into resonance-based sensors (Braun et al. 2009, Gil-Santos et al. 2010, Schmid et al. 2011) for biomolecular detection (reviewed in (Tamayo et al. 2013)). This approach exploits the nanomechanical properties (resonance frequency) of nanowire cantilevers as vibration propagates along the sensor. Capture of an analyte results in alteration of the vibrational pattern of the cantilever. Such systems offer label-free detection opportunities, as cantilever resonance frequency is proportional to analyte binding. This approach has been used to demonstrate the multiplexed label-free biodetection and quantitation of DNA in solution (McKendry et al. 2002). Hegner and colleagues (2012) have demonstrated label-free detection and quantification of α -synuclein in a phosphate-buffered solution. In this setup, the cantilever is clamped to the top of a piezo electric actuator inside the fluidic

chamber. A frequency generator causes resonance of the cantilevers which is detected by optical beam deflections. The α -synuclein monomer solution passes over the array, allowing binding to occur, and the resonance of the cantilever is recorded at multiple frequencies for subsequent data analysis. Nanomechanical biosensors have the potential to provide clinically relevant label-free detection of analytes, however the technology is still maturing. Approaches to analysing resonance frequency in axisymmetric nanowires (Gil-Santos et al. 2010) will help the technology to become more widely adapted to biosensing applications.

3.1.4.4 Nanowires in magnetic biosensors

Although the magnetic properties of micro- and nanoparticles have been used for the separation of target molecules from biological samples, magnetism is well-developed as an analysis mode for several biological assays (overviewed in (Muluneh & Issadore 2014)). Indeed, magnetic-based sensors are seen as one possible replacement for systems reliant on optical detection. For example, it is possible to label cells with magnetic particles in solution, followed by their detection by passing them over a magnetic detector where the magnetic signal is proportional to the amount of cell surface biomarker (Adams et al. 2008). Recently, a magneto-resistive biosensor was developed for the detection of a soluble ovarian cancer biomarker. The sandwich-ELISA-type system employed capture antibodies hybridised to a giant-magneto-resistance (GMR) sensor with the detection antibodies tagged with magnetic particles and showed improvements on the ELISA limit-of-detection (Hall et al. 2013).

Magnetic-barcoded nanowires represent another opportunity to add multiplexing capabilities to developing nanowire-based assays. Choi et al. (2005) demonstrated the synthesis of magnetically-encoded metallic nanowires through anodization of magnetic and non-magnetic metals in a porous alumina membrane. The ferromagnetism of these structures was altered by varying the proportion of magnetic and non-magnetic metals (elements such as Cobalt/Nickel and Gold, respectively), and it is believed that this tuneable property will aid in their application to multimodal sensing systems (Rosa et al. 2012, Vega et al. 2012).

Another potential advantage of integrating magnetic particles into diagnostic assay platforms is that it offers the chance to drastically simplify the signal output analysis required, compared to more conventional fluorescence-based assay systems. For example, it is not uncommon for clinical diagnostic laboratories to use fluorescence based assays consisting of more than 8 different fluorophores. The simultaneous use of so many

fluorophores at one time brings with it significant challenges in signal compensation, fluorophore bleed-through and false-positive signals. The use of magnetic particles in such systems could reduce complexity whilst maintaining assay resolution, and the knock-on effect of reducing overall assay cost could make the delivery of future biological assays to a wider market more feasible.

3.1.5 Flow cytometry: Application to nanowire analysis

Flow cytometry is an optical approach to interrogation of micron and sub-micron particles based on light scatter and absorption/emission properties. In a general flow cytometer setup, monochromatic light (e.g. a 405nm laser) is brought into contact with a sample via that flows in a fluidics chamber perpendicular to the incident light. If the sample contains a fluorophore, the fluorophore absorbs the incident monochromatic light (excitation radiation) and releases a burst of light (emission radiation), known as a pulse, at a longer wavelength. The scattered light pulse is collected by two detectors (forward scatter [FSC] and side scatter [SSC]) and filtered to emission sensors. Each pulse is digitised and displayed for analysis (Figure 3.3). Emitted light scatter pulses can be described in terms of wavelength, and pulse area, height and width. It is the analysis of these pulse parameters which form the cornerstone of all flow cytometric evaluations. Before analysis of test samples can begin, the functionality of the flow cytometer is calibrated by taking measurements from a standardised sample containing multiple fluorophores (such as the SPHERO™ 8-Peak Rainbow Beads). This ensures that the cytometer is capable of collecting the emitted radiation from potential fluorophores in test samples. Flow cytometric analysis can then be carried out by comparing test samples to a variety of biological (e.g. isotype control fluorescent antibodies) and assay component (e.g. reaction tube containing all assay components except the test sample) control samples. Flow cytometry has been extensively developed for the study of cell populations, and its high throughput capabilities (capable of recording up to 10,000 events per second) coupled with numerous wavelength filters mean that it is readily adapted to multiplexed applications (such as multicolour immuno-phenotyping). Additionally, the ability to gather data on millions of cells in a single sample statistically increases the chances of detecting rare cell types (such as lymphocyte sub-populations). To date, the vast majority of flow cytometric analysis has been focused on the interrogation of spherical objects (cells, microbeads). However, the axially restricted laminar flow setup means that flow cytometry could be an ideal tool for the analysis of anisotropic particles (already demonstrated for sperm cells and DNA chromosomes) such as nanowire populations.

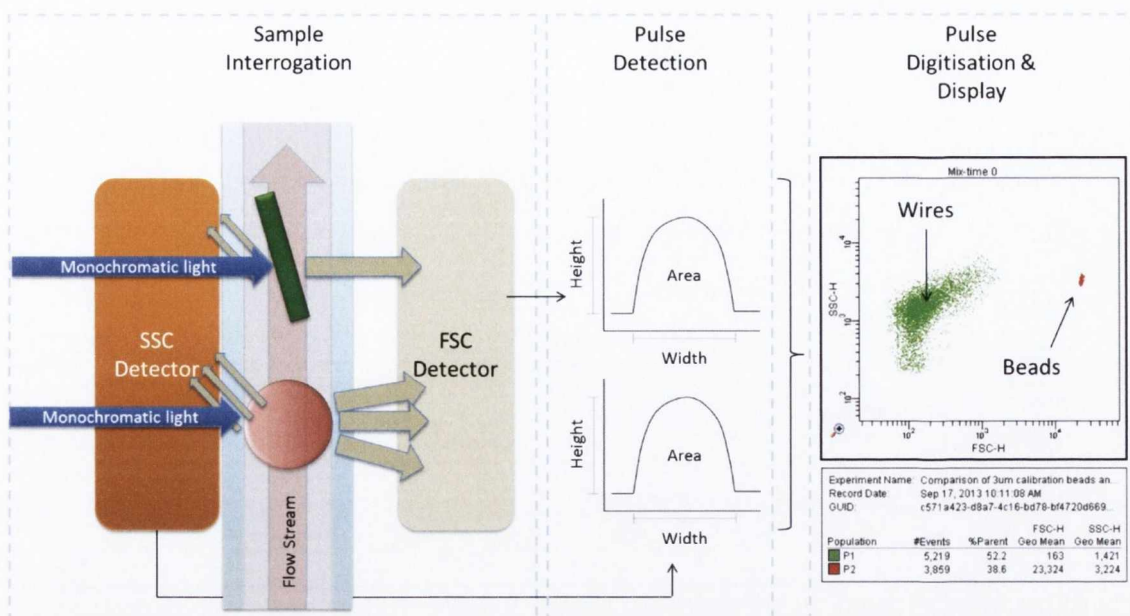


Figure 3.3 General flow cytometry setup. Sample interrogation: Micron and sub-micron particles such as beads (pink sphere) and wires (green cylinder) are passed in a laminar flow stream past an incident monochromatic light path (blue arrows). **Pulse Detection:** scattered light is recorded by forward scatter (FSC) and side scatter (SSC) detectors as an analogue pulse with measurable wavelength, height, area and width. **Pulse Digitisation & Display:** Cytometer software digitises the pulse and each parameter can be interrogated using linear or logarithmic plots.

As illustrated in the above section, the design process involved in developing a nanomaterial-based probe for diagnostic applications is a dynamic one and must assimilate considerations on synthesis, functionalisation, technological development and system integration. The examples used to exemplify the successful application of nanowires to each of the detection modalities underlines the fact that nanowire-based technologies are maturing to a level where they can start to deliver clinical benefit, both in terms of increased sensitivity and reduced assay time and resources. However, there are limitations to consider within each developing technological platform, not least the hardware integration/processing burden required to perform these novel assays can sometimes not compete with the simplicity of existing clinically-used platforms. It is therefore necessary to continue development of nanomaterial-based assays which integrate into existing, clinically useable platforms.

3.1.6 Chapter 3 synopsis graphic

Within this chapter, the initial development of nanoprobe for detection of soluble cancer biomarker sErbB2 will be demonstrated. The validation of the nanoprobe as a proof-of-concept device comprises three main steps, namely; nanomaterial characterisation, functionalisation with antibody and detection of a purified analyte in a single analyte system (synopsised in Figure 3.4).

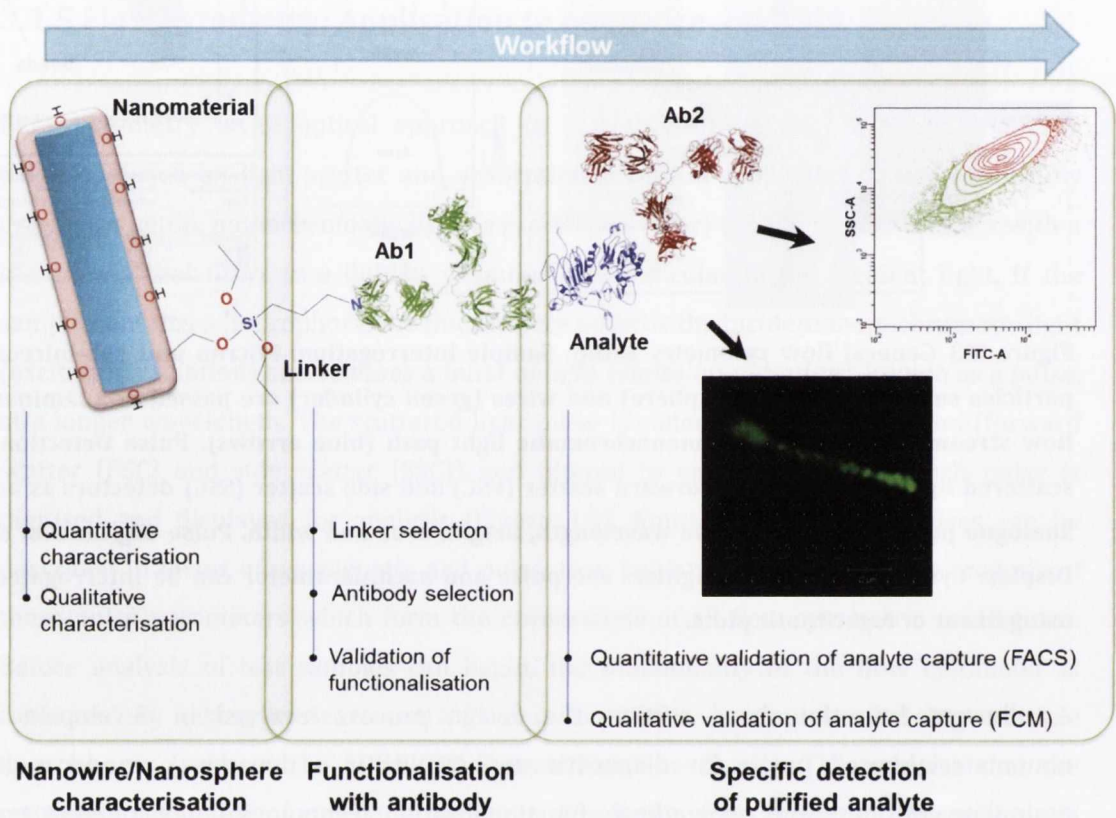


Figure 3.4 Schematic of development of nanoprobe for detection of soluble cancer biomarkers.Workflow: Nanomaterial characterisation: Nanowires (light blue cylinder) or nanospheres (not shown) are quantitatively characterised via a number of techniques including flow cytometry (FACS) and nanoparticle tracking analysis. Qualitative assessment of nanomaterials is carried out by electron microscopy and atomic force microscopy. Functionalisation with antibody: Target-specific green-fluorescent antibodies (Ab1) are conjugated to the nanomaterial surface via the APTES linker molecule. Validation of functionalisation is achieved through use of fluorescent confocal microscopy (FCM), atomic force microscopy, Helium-ion microscopy, nanoparticle tracking analysis, spectroscopy and gel electrophoresis. Specific detection of purified analyte: Following incubation of the functional nanomaterial with the target analyte, detection of an analyte (sErbB2) is achieved via application of a complementary, target-specific fluorescent antibody (Ab2). Specificity for the analyte is assessed by testing the system using another soluble protein, bovine serum albumin. Analyte capture is assessed quantitatively and qualitatively via FACS and FCM, respectively.

3.2 Results

3.2.1 Representative cell images for cell lines used in experiments.

Representative images of each cell line used in experiments are shown below, see Figure 3.5 and 3.6. All cell lines were maintained in the recommended nutrient media, passaged at ~80% confluence and cell lines were kept below passage 20. Results show that the cells are healthy and have the expected morphology indicative of the cell line.

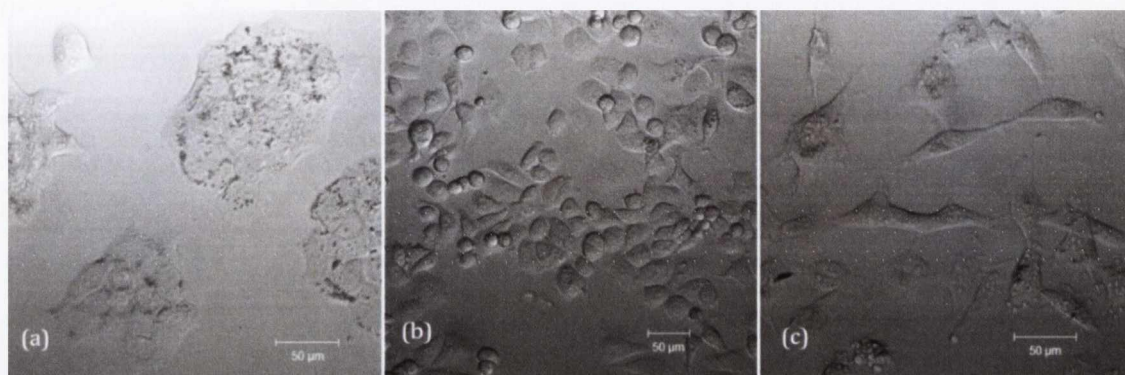


Figure 3.5 Breast cancer cell lines; (a) BT-474, (b) SK-BR-3 & (c) MDA-MB-231, were cultivated for use in experiments as detailed in the Materials and Methods Chapter. Cell morphology images were obtained using a bright-field microscope (Zeiss, Germany) at 20X or 40X magnification.

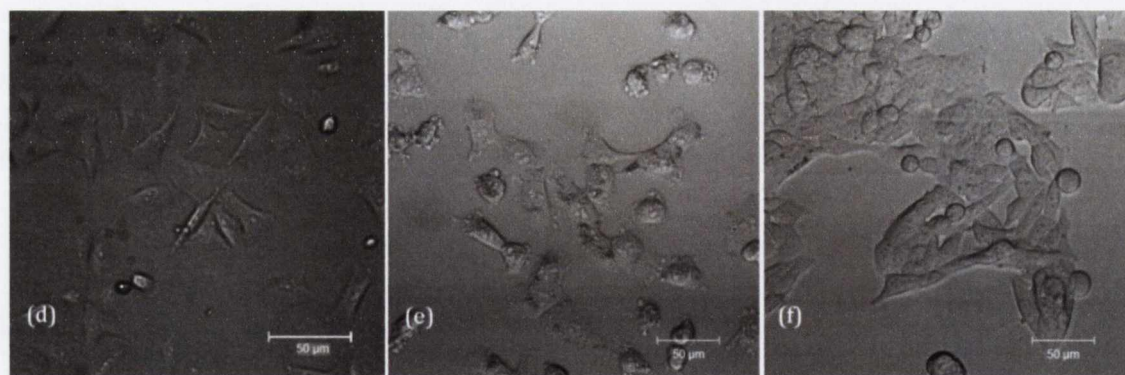


Figure 3.6 Lung cancer derived cell lines; (d) A549, (e) H596 & (f) H520, were cultivated for use in experiments as detailed in the Materials and Methods Chapter. Cell morphology images were obtained using a brightfield microscope (Zeiss, Germany) at 20X or 40X magnification.

3.2.2 Western Blotting assessment of ErbB2 status in cancer cell lines

SDS-PAGE gel electrophoresis and Western Blot were used to separate, identify and visualise ErbB2 protein content in six cell lines. Actin protein content was used to normalise for equal sample loading. The results in Figure 3.7 (a) show that the breast cancer cell lines BT-474 and SK-BR-3 show high expression of ErbB2 in comparison to MDA-MB-231, A549 and H596 cancer cell lines which expressed similar levels of ErbB2 which were relatively low compared to BT-474 and SK-BR-3 cell lines. The lung cancer cell line, H520, shows trace levels of ErbB2. Figure 3.7 (b) is generated from the data shown in figure 3.7 (a) and calculates the relative intensity of ErbB2 to Actin in the six cell lines tested. The results show that BT-474 and SK-BR-3 show the highest ErbB2:Actin ratio, MDA-MB-231, A549 and H596 cell lines had similarly low levels, and H520 had trace levels of ErbB2 compared to Actin expression. This characterisation was important in confirming that our starting cell cultures had the expected ErbB2 status, and served as a reference for further studies (e.g. confocal microscopy) related to the cellular expression of ErbB2.

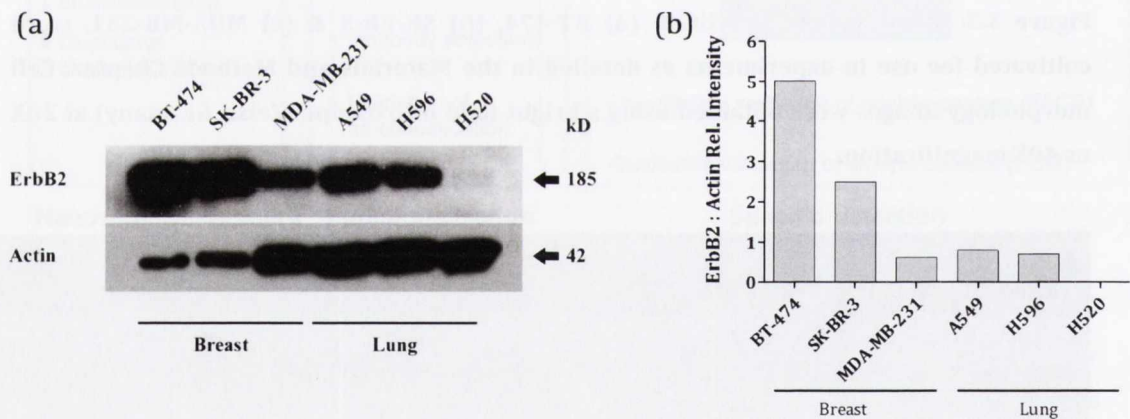


Figure 3.7 Western blot assessment of ErbB2 status in six breast and lung cancer cell lines. (a) Western blot using the ErbB2 (29D8) rabbit mAb shows bands appearing in five of the six cell lines tested (all except H520). The same blot probed with the Anti- β -Actin mouse mAb indicates the relative amounts of actin present in each sample. Note that for BT-474 and SK-BR-3 cell lines, the cell lysate was diluted prior to loading in the gel, compared to the other cell lines. This is reflected in the visibly decreased actin intensity of both cell lines in the lower panel. (b) The intensity of each band in (a) was calculated using ImageJ software, and the relative intensity for each cell line was calculated and plotted. This data confirms that BT-474 and SK-BR-3 cell lines are high expressers of ErbB2 compared to other cell lines. Cell line H520 expresses trace levels of ErbB2.

3.2.3 ELISA assessment of ErbB2 status in cancer cell lines

ELISA was used to assess the concentration of total ErbB2 protein in the cell lysate of six cancer cell lines. The quantification was carried out in triplicate, using SKOV3 cell lysate as a known positive control. The data is expressed as picogram (pg) ErbB2 per microgram (μ g) total protein, quantified by Bradford protein assay. The data generated from ELISA assessment of these cell lines closely correlates with the Western blot data previously shown.

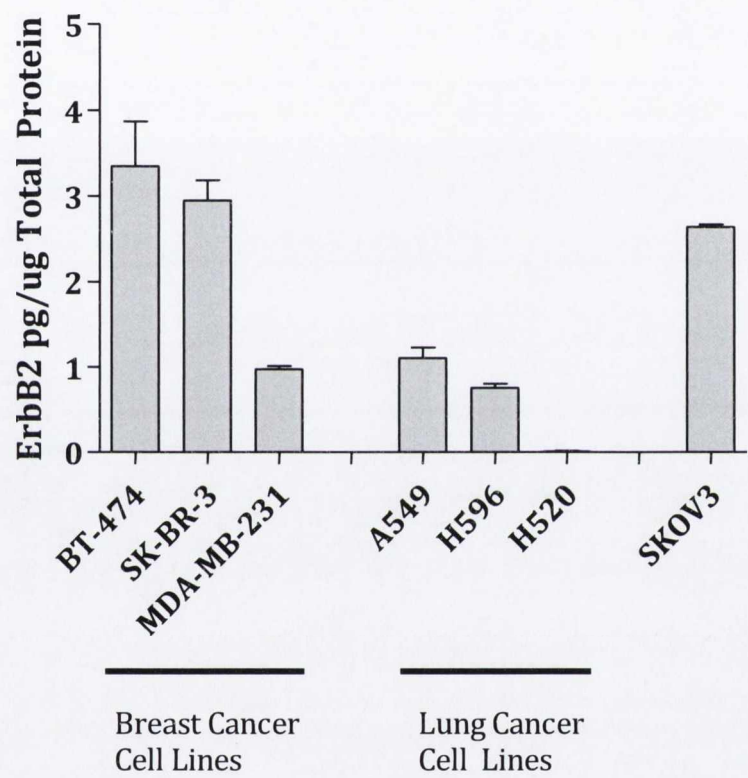


Figure 3.8 Quantification of total ErbB2 per unit total protein in breast and lung cancer cell lysates. The data show that BT-474 and SK-BR-3 breast cancer cell lines contain the highest concentration of ErbB2 per total protein. MDA-MB-231, A549 and H596 cell lines exhibited comparable levels of ErbB2, whilst H520 showed no detectable level of ErbB2. SKOV3 demonstrated a strong positive ErbB2 signal.

3.2.4 Assessment of fluorescent ErbB2 antibody performance using cancer cell lines

Breast cancer cell lines were immunostained with anti-CD340-AlexaFluor488 (Biolegend, USA). Two ErbB2-positive breast cancer cell lines (BT-474 and SK-BR-3) and one ErbB2-negative breast cancer cell line (MDA-MB-231) were used. The results indicate that the anti-CD340-AlexaFluor488 antibody did bind to BT-474 and SK-BR-3 cell lines but did not bind to the MDA-MB-231 cell line. Taking the Western blot and ELISA data on ErbB2 expression in breast cancer cell lines into account, the conclusion can be made that anti-CD340-AlexaFluor488 binds to the cell lines which express high levels of ErbB2 but not to the cell line with low expression of ErbB2.

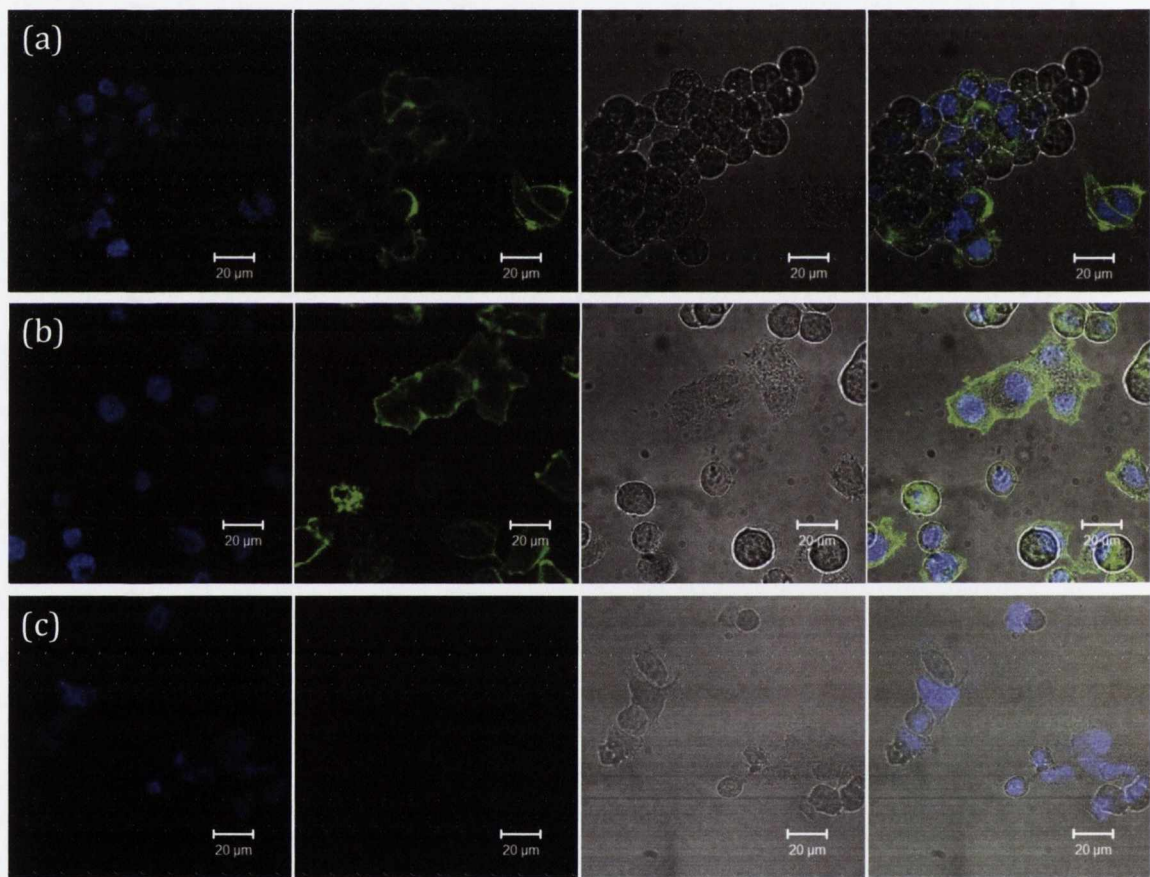


Figure 3.9 Immunostaining of breast cancer cell lines using fluorescent anti-CD340-AlexaFluor488. Confocal microscopic images of immunostained (a) BT-474, (b) SK-BR-3 and (c) MDA-MB-231 breast cancer cell lines. Column 1: Hoescht 34580 nuclear stain. Column 2: anti-CD340-AlexaFluor488. Column 3: brightfield image. Column 4: merged image. Strong green fluorescent signal (AlexaFluor488) from BT-474 and SK-BR-3 cell lines is visible whilst no green signal from the MDA-MB-231 cell line is detected. These results indicate high fluorescent staining on BT-474 and SK-BR-3 cell lines.

Lung cancer cell lines were also immunostained with anti-CD340-AlexaFluor488 as above. Two lung cancer cell lines (A549 & H596) with low ErbB2 expression and one ErbB2-negative breast cancer cell line (H520) were immunostained. The results indicate no detectable fluorescent signal in any of the lung cancer cell lines tested, indicating the absence of the anti-CD340-AlexaFluor488 on the lung cancer cells. Western blot and ELISA data confirmed that the ErbB2 expression levels in A549 and H596 cell lines were similar to MDA-MB-231 levels, whilst the H520 cell line showed no ErbB2 expression. Given that MDA-MB-231 immunostaining with anti-CD340-AlexaFluor488 did not show a fluorescent signal, and A549, H596 and MDA-MB-231 cell lines have similar ErbB2 expression profiles (see Western blot and ELISA data) the conclusion is reached that the tested antibody does not provide sufficient signals in cells with low ErbB2 expression levels.

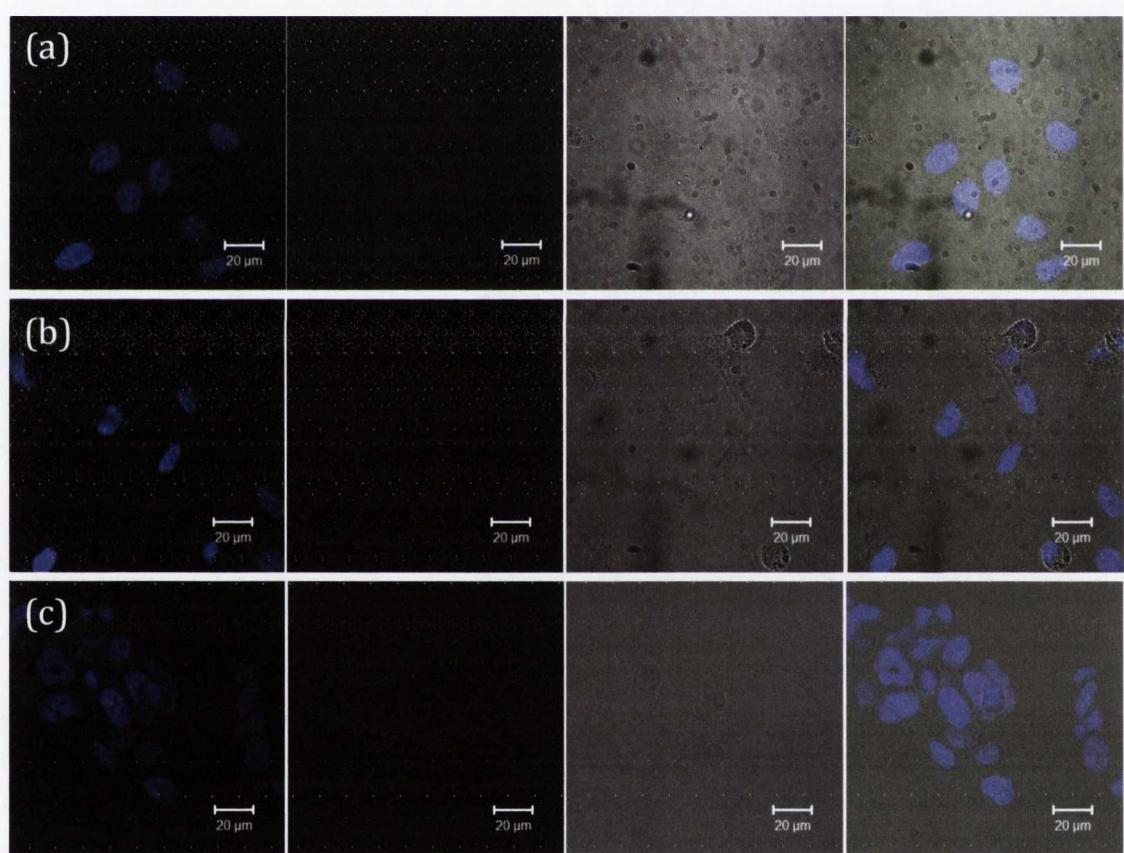


Figure 3.10 Immunostaining of lung cancer cell lines using fluorescent anti-CD340-AlexaFluor488. Confocal microscopic images of immunostained (a) A549, (b) H596 and (c) H520 lung cancer cell lines. Column 1: Hoescht 34580 nuclear stain. Column 2: anti-CD340-AlexaFluor488. Column 3: brightfield image. Column 4: merged image. Results show no green fluorescent signal (AlexaFluor488) from any of the cell lines indicating the absence of anti-CD340-AlexaFluor488 on the cells following the immunostaining procedure.

3.2.5 Microscopic analysis of bulk nanomaterials

3.2.5.1 Microscopic analysis of silica nanowires

Qualitative assessment of silica nanowires (see section 2.2.4.1) was made by Helium-ion microscopy (HIM) (refer to section 2.4 for synopsis of techniques), see figure 3.11 (a). HIM images show the nanowires to be of varying lengths and diameters, and smaller fragments of similar density were also visible in the preparation. The nanowires were a range of lengths, and some nanowires exhibited a branched structure (denoted with red arrows in below image). Nanowire aggregates were also visible (denoted by green arrow in below image). Using HIM, it was possible to achieve good contrast for imaging the silica nanowires without extensive post-capture image processing. Frequency distribution data (b) calculated from analysis of HIM images show that the wires ranged in length from 1.3 - 5.6 μm , having a mean length of 2.79 μm and a large standard deviation from mean ($\pm 1.13 \mu\text{m}$).

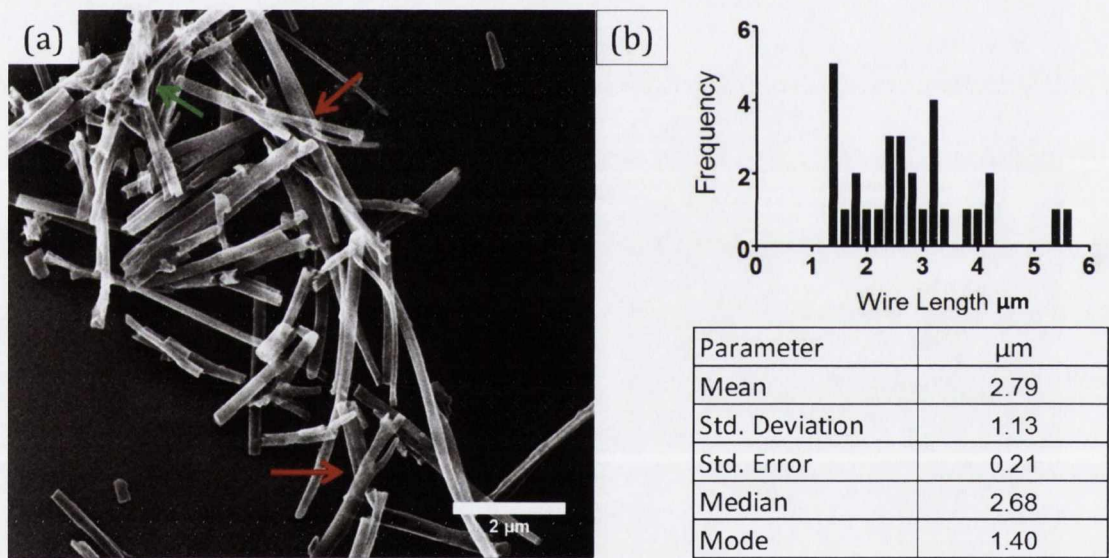


Figure 3.11 (a) HIM image of silica nanowires. Nanowires vary in length and thickness and nanowire fragments are also visible. Some of the nanowires exhibited a branched structure (indicated by the red arrows) and nanowire aggregates were also visible (green arrow) (b) frequency distribution of wires. (n=30). Scale bar = 2 μm .

Qualitative assessment of silica nanowires was further made by TEM (Figure 3.12 (a) & (b)). TEM analysis noted some magnetite deposits embedded in the silica wire. Magnification of one nanowire section (b) showed that the diameter of the nanowire at that point was about 100 nm.

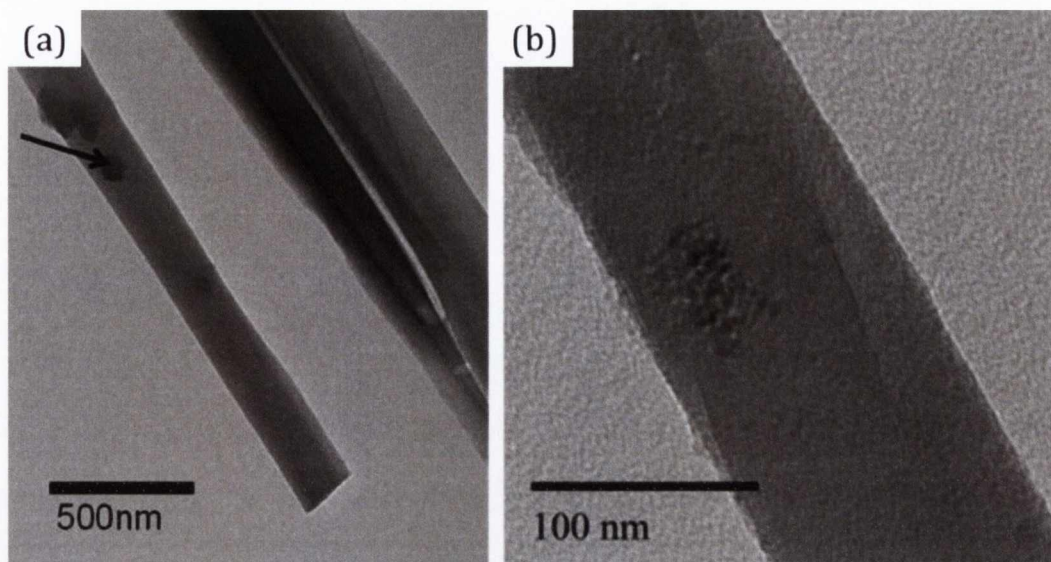


Figure 3.12 TEM of silica nanowires. (a) Arrow denotes embedded magnetite (Fe_3O_4) incorporated during synthesis. (b) Enlarged image of nanowire shows diameter at ~ 100 nm.

Microscopic analysis of silica nanowires using HIM and TEM showed a nanowire population with length distribution between $1.3 - 5.6 \mu\text{m}$, of varying diameter and with a small degree of branching. HIM was found to provide adequate contrast to allow for size distribution analysis of silica nanowires.

3.2.5.2 Microscopic analysis of CoNi nanowires

Qualitative assessment of CoNi nanowires was made by SEM (Figure 3.13 (a)). SEM images showed straight CoNi nanowires which were highly uniform, both in terms of length and diameter. No branching was visible in this nanowire population, and the preparation appeared free of aggregates. Frequency distribution data (b) calculated from SEM show highly uniform wire lengths with a mean length of $9.65 \mu\text{m}$ and a standard deviation from mean of $1.35 \mu\text{m}$. The standard deviation in length observed in the CoNi nanowires was, proportionally, much smaller than that observed in the silica nanowire population. The uniformity of the CoNi nanowires was greater than that of the silica nanowires. This is as a result of the difference in the synthesis processes between the two materials. CoNi nanowires were grown within an alumina membrane (offering a rigid template which helps to confine the nanowire growth to one axis) whereas the silica nanowires were produced using a sol-gel preparation, which allows for branching to occur on the nanowires. This accounts for the difference in geometry between the nanowire types

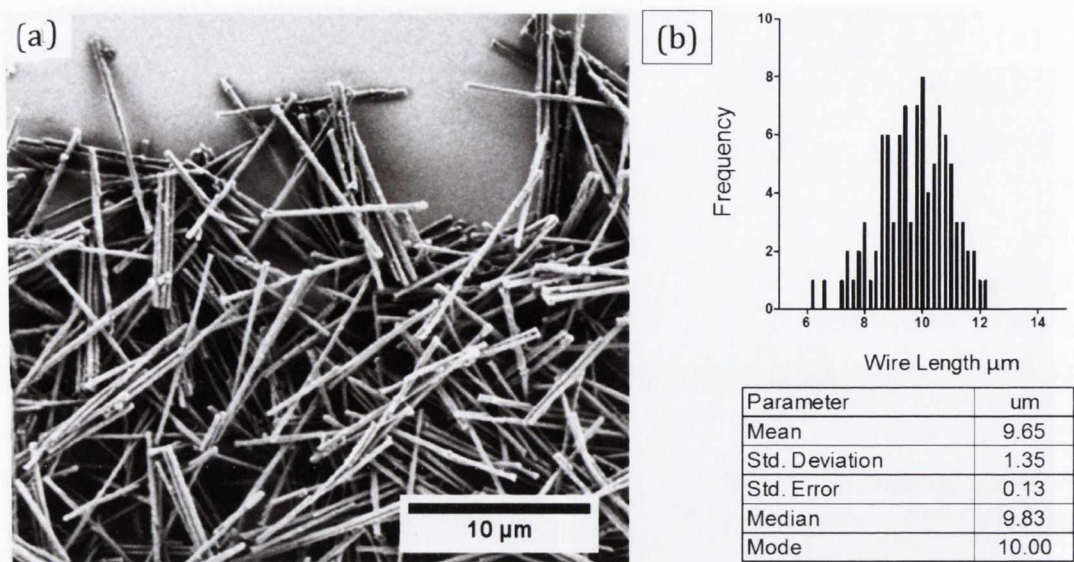
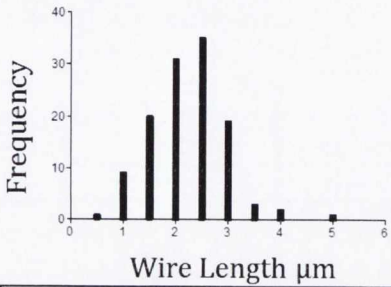
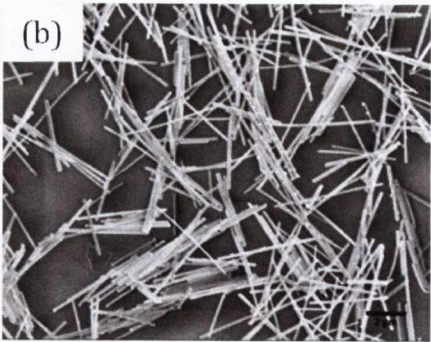
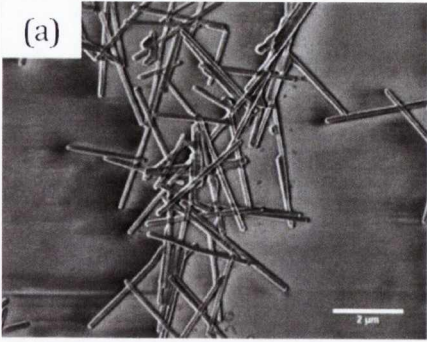


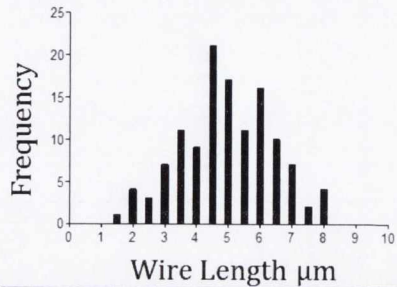
Figure 3.13 SEM of CoNi nanowires. Frequency distribution data (b) was calculated using GraphPad Prism software.

3.2.5.3 Microscopic analysis of Ag nanowires

Qualitative assessment of Ag nanowires was made by SEM (Figure 3.14). Ag nanowires were Au-sputter-coated prior to imaging in order to prevent charging of the nanowires, a phenomenon which interferes with SEM. Frequency distribution data (located directly beneath each individual representative image for the four Ag nanowire populations) was calculated from 100 measurements. The smaller Ag nanowire batches (nanowires (a) and (b) with mean length of 2.23 µm and 4.96 µm, respectively) were straight and length distribution data showed that standard deviation from the mean to be 0.69 µm and 1.44 µm respectively. Images of the longer Ag nanowire batches (nanowires (c) and (d) with mean length of 22.69 µm and 35.38 µm, respectively) showed very long nanowires, some of which had displayed occasional bending along their lengths. For both of the larger nanowire populations, the mode size was closer to the lower end of the size scale within each measured population, whereas for the smaller nanowire populations, the mode fell closer to the centre of the size distribution data. It is possible that sample (c) and (d) were more prone to breakage, due to their length.



Parameter	μm
Mean	2.23
Std. Deviation	0.69
Std. Error	0.06
Median	2.25
Mode	2.50



Parameter	μm
Mean	4.96
Std. Deviation	1.44
Std. Error	0.13
Median	5.09
Mode	4.50

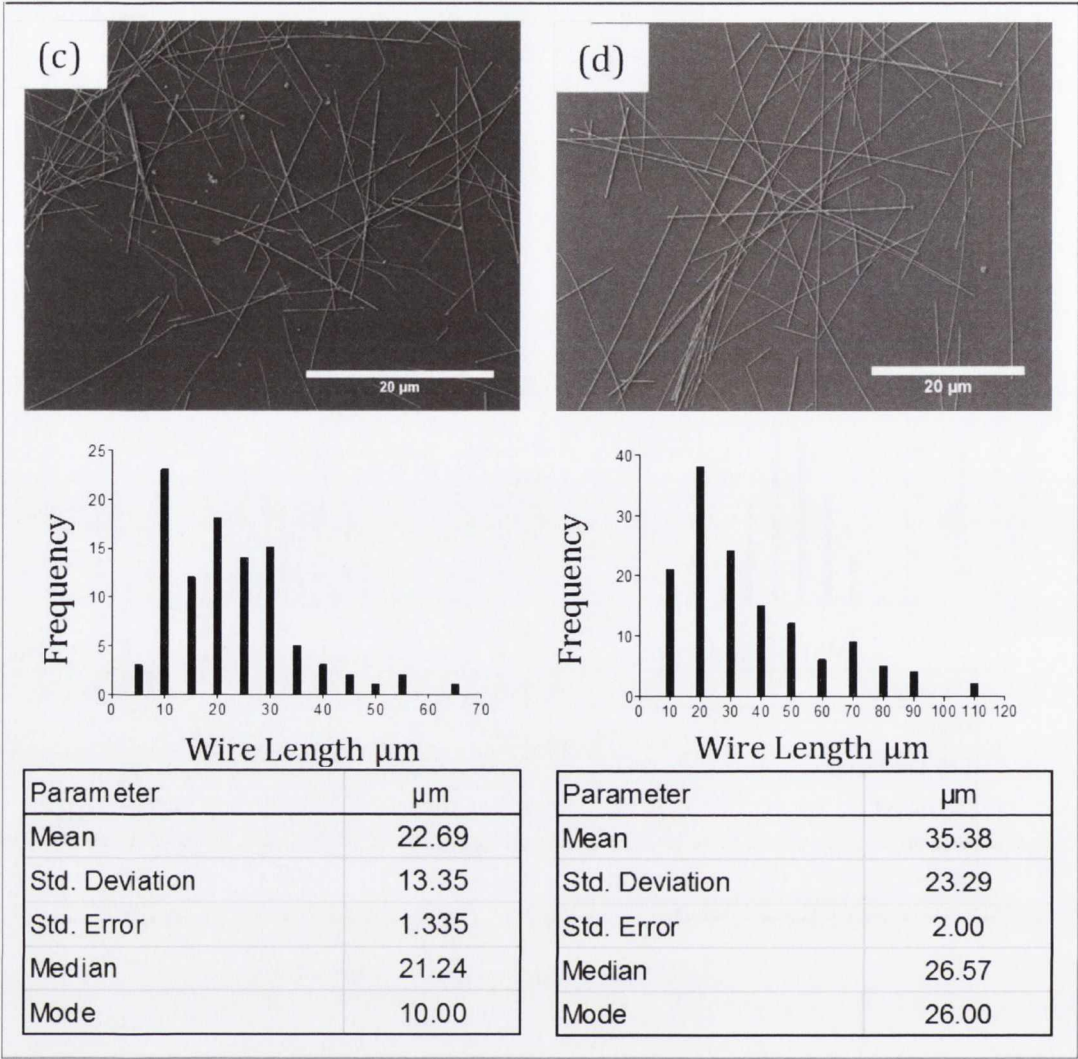


Figure 3.14 Size distribution determination of Ag nanowires by SEM. Four Ag nanowire batches (a-d) were imaged with SEM and nanowire length distribution was calculated. Mean length ($\pm$ Std. Deviation & Std. Error), median length and mode length values (μm) were measured and calculated for each batch of nanowires ($n=100$) using GraphPad Prism software.

3.2.5.4 Microscopic analysis of polystyrene microspheres

Qualitative assessment of polystyrene microspheres was made by SEM with representative images provided in figure 3.15 (a) – (f). Frequency distribution data of sphere diameter showed microspheres with high uniformity and small standard deviation. Despite the evident differences in the deposition patterns due to the electron microscopy preparation steps, it was still possible to obtain reliable measurements reflecting the data sheet provided by the supplier (See Appendix 2).

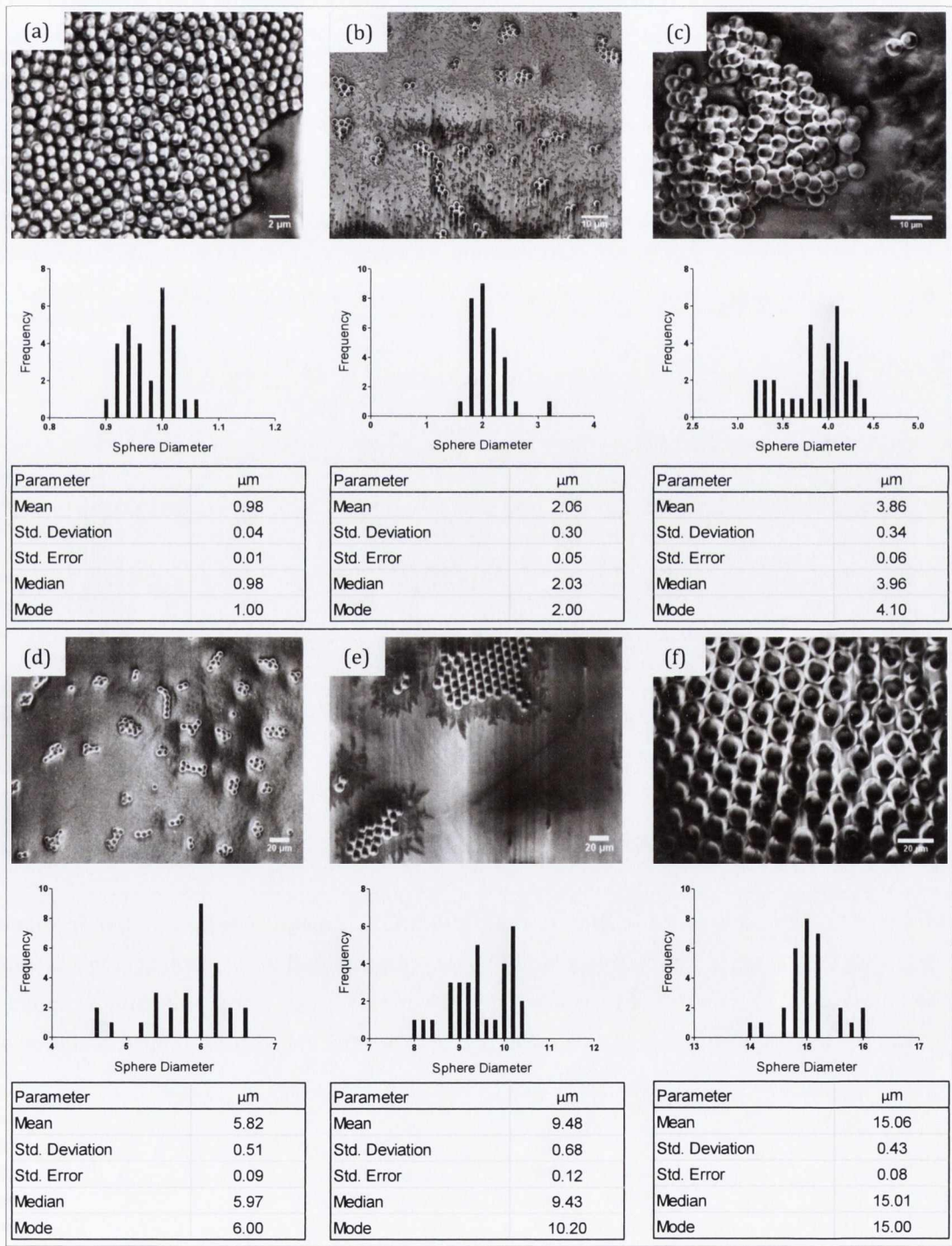


Figure 3.15 Sizing distribution determination of polystyrene spheres by SEM. Six polystyrene sphere batches (a-f) were imaged with SEM and diameter size distribution was measured using ImageJ. Mean diameter ($\pm$ Std. Deviation & Std. Error), median diameter and mode diameter values (μm) were obtained for each batch of spheres ($n=30$) using GraphPad Prism software.

3.2.5.5 Microscopic analysis of silica nanospheres

Qualitative assessment of silica nanospheres was assessed using SEM (Figure 3.16). Nanospheres appeared uniform in shape and nanosphere diameter distribution data showed mean nanosphere diameter to be 99.5 nm. The standard deviation in the sample was quite low at ± 9.9 nm. The uniformity of the nanospheres as determined by SEM was supported by data gained from analysis of the silica nanospheres using the Nanosight NS500 Nanoparticle Tracking Analysis software, which confirmed that the suspension was monodisperse and that the hydrodynamic radius was similar to the nanosphere core measurements gained from SEM (see section 3.2.6.2 for Nanosight data).

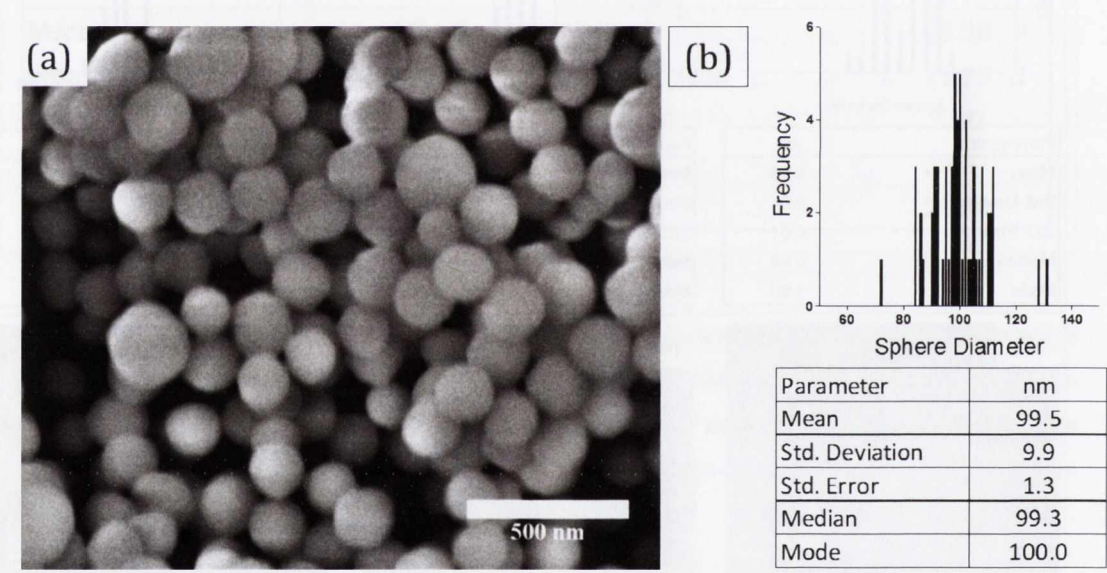


Figure 3.16 SEM of silica nanospheres. (a) Nanospheres appear to be sphere-like in shape. Scale bar = 500 nm. The sample was highly uniform and free from aggregates and contamination with debris. (b) Diameter distribution data was calculated using GraphPad Prism software and showed the mean sphere size to be 99.5 nm with a standard deviation of 9.9 nm.

3.2.6 Functionalisation of nanomaterials with antibodies

3.2.6.1 Determination of stock concentration of nanowires using FACS

Figure 3.17 (a) shows the background signal obtained from running filtered water (theoretically, containing no particles greater than 100 nm in any dimension) through the flow cytometry system. These recorded events are partly due to “electronic” noise in the system and are a result of increasing the voltage across the detectors in order to increase detector sensitivity, and may also be due to some particles which may have remained after filtration. In this figure, the voltage to the sensor has been increased so that the filtered water begins to register events above the side-scatter (SSC) threshold (demarcated by the visible horizontal cut-off line in each plot). The data gained from analysing the nanowire dispersions at two different dilutions are shown in figure 3.17 (b) & (c) at 1:5000 and 1:1000 respectively. The data shows a dramatic increase in event count over the same time, compared to the filtered water sample. This shows that the nanowires are only visible when the system voltage has been increased to an extent that the filtered water sample alone starts to generate events above the side-scatter (SSC) threshold. In addition there is a concentration dependent increase in the number of measured events in the nanowire samples with a decrease in dilution factor. As the flow rate (number of $\mu\text{l}/\text{min}$) of the cytometer had been previously calculated, and by measuring the sample of nanowires for a defined period of time (ie. 3 min), the number of events per ml could be calculated. This number was then multiplied by the dilution factor to estimate the nanowire concentration of the sample.

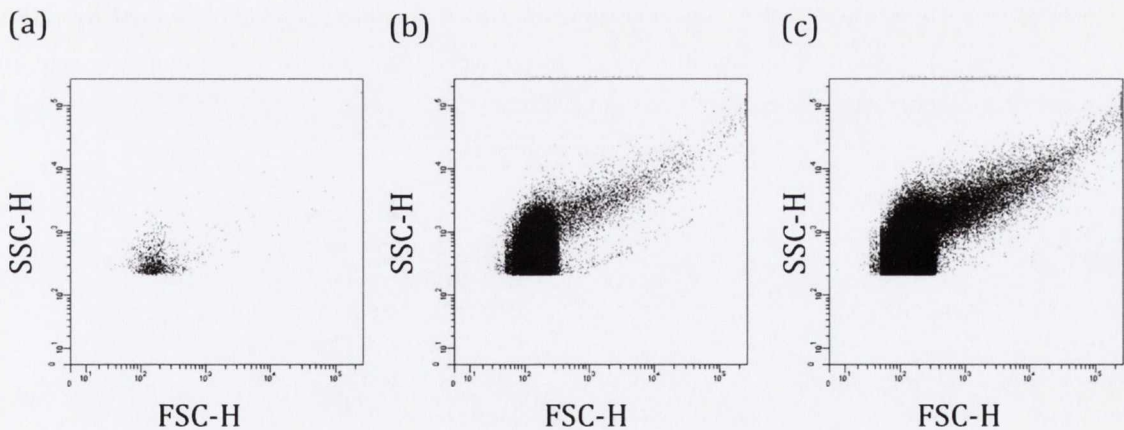


Figure 3.17 Flow cytometric analysis of nanowires. Two dilutions of nanowires, 1:5000 (b) and 1:1000 (c), were compared to filtered water (a). A wide scatter profile supports the qualitative data which shows wires of various length and uniformity. The concentration of stock nanowires in this example was calculated as approximately 1.89×10^9 particles/ml based on the method outlined in the Materials and Methods chapter.

3.2.6.2 Determination of stock concentration of silica nanoparticles using NanoSight NS500

The stock concentration for each nanoparticle dispersion (particles/ml) was determined using the NanoSight NS500 (NanoSight, Wiltshire, UK) with the on-board Nanoparticle Tracking Analysis software (Figure 3.18). The batch average results show that the nanoparticle suspension was free from aggregates, contained only one significant size population (i.e. only one peak was visible in the graph) and the mode nanoparticle diameter was 106 nm based on six consecutive measurements. The NS500 on-board software calculated the total concentration of the suspension to be 3.52e+13 particles/ml.

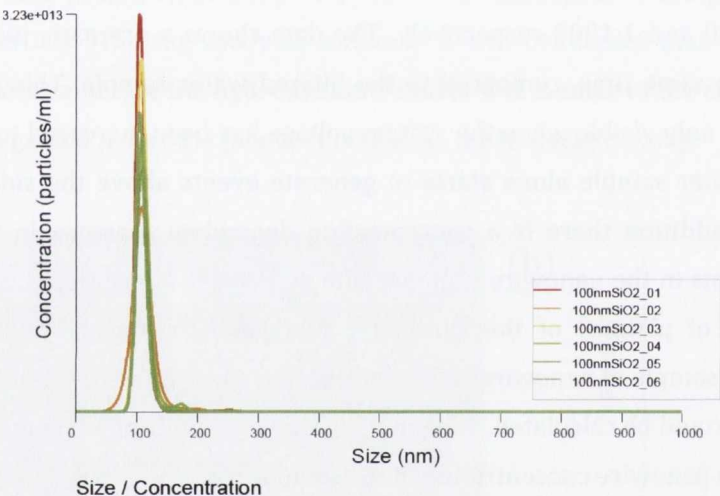


Figure 3.18 Size and stock concentration of silica nanospheres as determined by NS500. Silica spheres diluted in sterile filtered water were assessed for hydrodynamic size and stock concentration using the NanoSight NS500.

BATCH AVERAGE RESULTS:	
FTLA Size Distribution:	Mean: 109.3 +/- 0.5 nm
	Mode: 106 +/- 0.8 nm
	SD: 15.2 +/- 1.6 nm
	D10: 94 +/- 1.6 nm
	D50: 106.0 +/- 0.7 nm
	D90: 123 +/- 1.7 nm
Dilution factor:	1000000
Total Concentration:	3.52e+013 particles/ml
Total Completed Tracks: 7908	
Average Drift Velocity: 750 nm/s	

Figure 3.19 Batch average results for stock measurements of silica nanospheres using NanoSight NS500. The mode hydrodynamic diameter distribution was calculated to be 106 nm +/- 0.8 nm and the total concentration was calculated at 3.52e+13 particles/ml.

3.2.6.3 Observation of EDC reaction temperature by flow cytometric approach

The effect of heat (generated by sonication of sample) on the efficiency of EDC coupling reaction of antibodies to the silanised nanowire surface was assessed by measuring the %Parent of the reaction sample containing a fluorescent signal (indicating antibody binding to nanomaterial surfaces) at 30 min time-points. The data (Figure 3.20) shows that an increase in reaction temperature (above 32° C) correlates with a loss of fluorescent signal from nanowires. Even when additional EDC was added to the reaction (120 min), the sample did not recover fluorescent signal. A reduction in temperature upon addition of ice correlated with a recovery of fluorescent signal in the nanowire population.

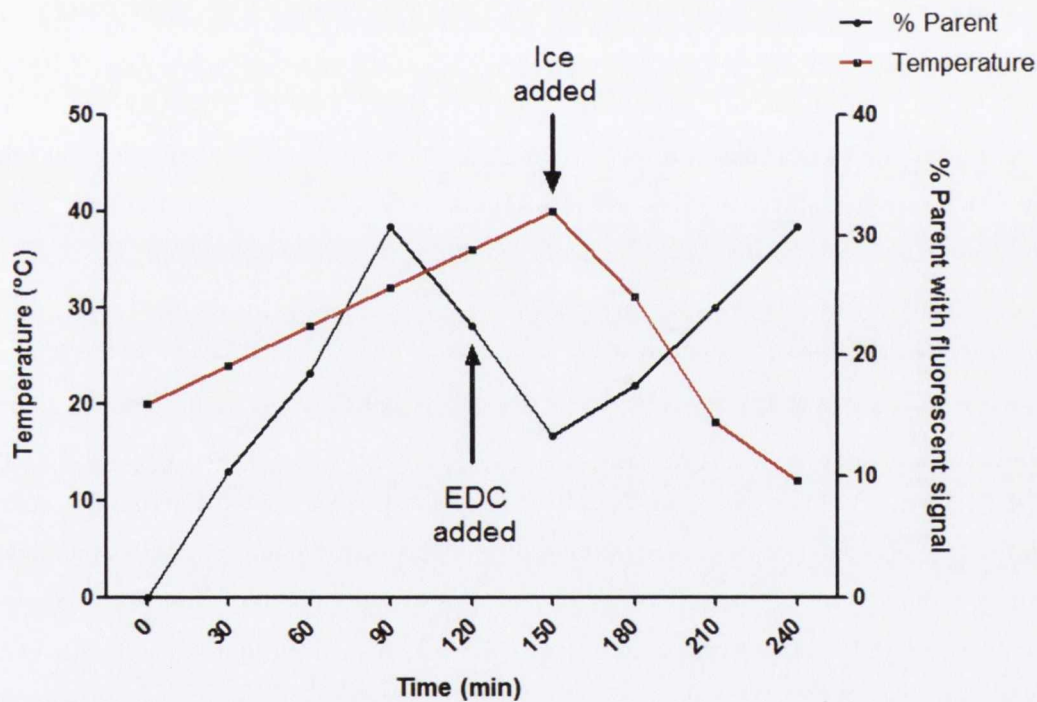


Figure 3.20 The effect of temperature on EDC-driven antibody coupling reaction. An initial rise in temperature (0 - 90 min) did not adversely impact the fluorescent signal in the nanowire population. Temperatures above 30°C correlate with a decrease in %Parent with a fluorescent signal, indicating the dissociation of fluorophores from the nanowire surface.

It is interesting to note that sonication itself appears not to be detrimental to the coupling of antibodies to the nanowire surface. This is supported by the steep rise in %Parent fluorescent signal for the first 90 min of the reaction. By measuring the %Parent of the nanomaterial population containing a fluorescent signal at regular intervals, flow

cytometry can be used to assess nanowire-antibody conjugation efficiency over time. Following these observations, consequent reactions were maintained below 30°C.

High temperatures appear to affect the stability of the EDC-driven antibody coupling reaction. Since EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) is usually stored and stable at -20 Celsius, exposure of EDC to high temperature may cause the inactivation of EDC. Although this phenomenon was not investigated further in this thesis, it is in line with findings from another group which studied the effect of temperature on activation efficiency of EDC, who found that increasing the temperature above 25 Celsius substantially decreased the activation efficiency of EDC (Xia et al. 2013).

3.2.7 Characterisation of antibody-conjugated nanowires

A number of characterisation techniques (namely, absorbance spectroscopy, gel electrophoresis and nanosight tracking analysis ; see section 2.4 for synopsis of techniques used) used for examining antibody attachment to nanospheres are not suitable for the characterisation of functionalised nanowires. As a result, several other approaches (atomic force microscopy and Helium-ion microscopy) were implemented as alternative methods for assessing the quality of nanowire functionalisation.

3.2.7.1 Fluorescent microscopy

Fluorescent confocal microscopy was used to optically examine the antibody-functionalised nanowires (Figure 3.21). Imaging of non-functionalised (Figure 3.21 (a), panel (i) below), fluorescent antibody-functionalised (Figure 3.21 (a), panel (ii) below) and non-fluorescent antibody-functionalised (Figure 3.21 (a), panel (iii) below) nanowire samples showed a marked difference in the optical signal coming from each nanowire sample. The mean fluorescence intensity (MFI) was measured along the length of the nanowire (Figure 3.21 (b), below) for each sample and clearly shows an increased signal in the fluorescent antibody-functionalised nanowire sample (ii). The non-functionalised nanowire sample and the non-fluorescent antibody-functionalised nanowire sample showed negligible optical signal as denoted by the calculated MFI close to 0 for both samples (Figure 3.21 (c), below). This data confirms the presence of antibody on the nanowire surface and allows the visualisation of the distribution of fluorescent antibodies on the nanowire surface, through assessment of the optical signal emitted from the fluorescent antibody.

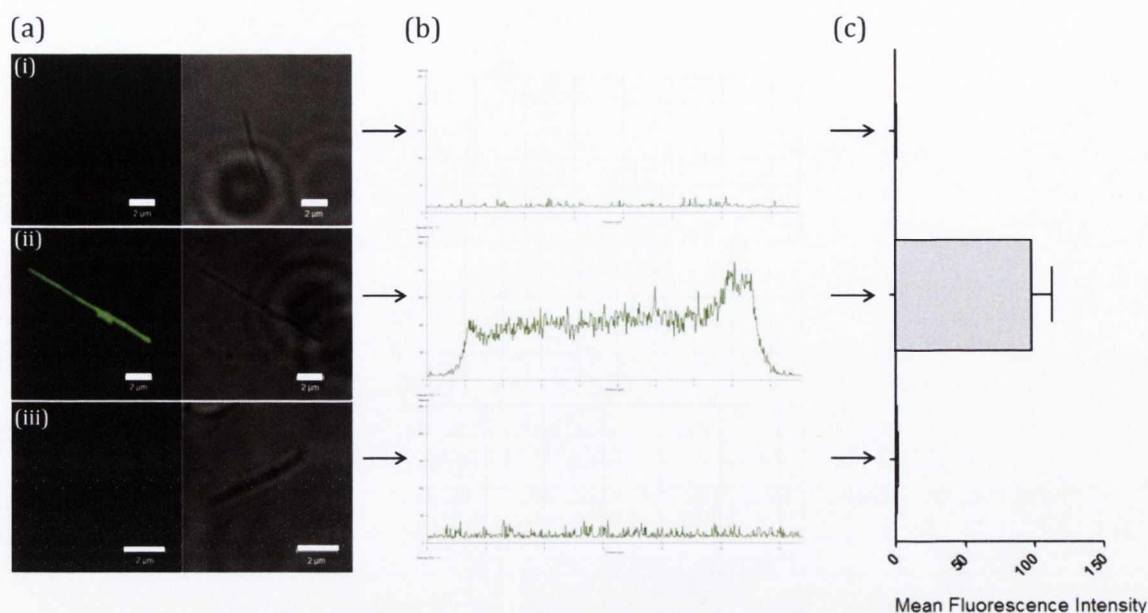
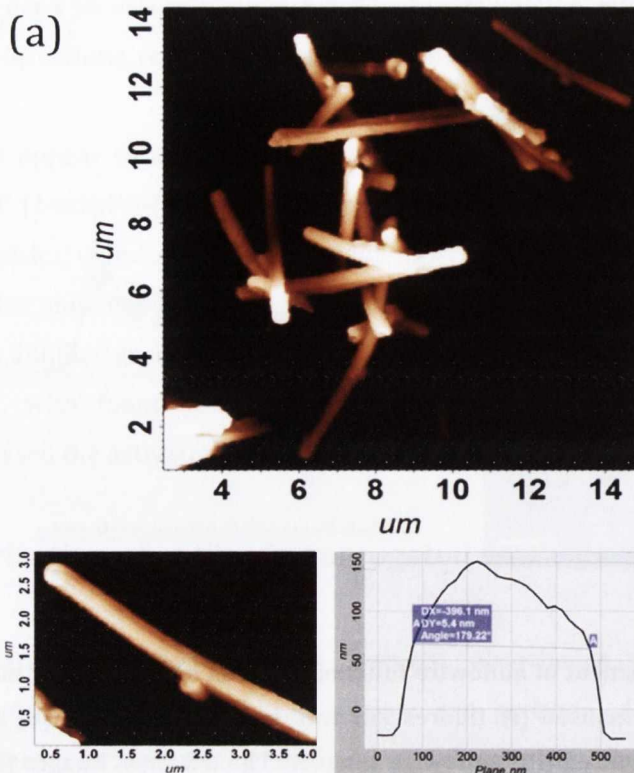


Figure 3.21 Assessment of nanowire functionalisation by fluorescent microscopy. (a) Panel shows non-functionalised (i), fluorescent antibody-functionalised (ii) and non-fluorescent antibody-functionalised (iii) nanowire samples. The left-most images (labelled: (i), (ii) and (iii) above) are the fluorescent channel images, whilst the accompanying images (right, in (a) above) are the brightfield images for the same samples. (b) The line profile mean fluorescence intensity of each sample was measured on each wire's length using confocal microscopy. (c) Mean fluorescent intensity values for each nanowire sample preparation (n=20). Scale bar = 2 μm.

3.2.7.2 Atomic force microscopy

Atomic force microscopy was used to physically confirm nanowire functionalisation (Figure 3.22). Non-functionalised (Figure 3.22 (a), below) and functionalised (Figure 3.22 (b), below) nanowires were compared. Following the conjugation of antibody to the nanowire, a change in the surface roughness is noticeable (Figure 3.22 (b), left and upper right) compared to non-functionalised nanowires (Figure 3.22 (a), left and upper right). This is indicative of the presence of antibody on the wire surface. The diameter of stock and functionalised nanowires could be measured and an increase in diameter after functionalisation was noted (in this example from 396 nm to 500 nm). However taking into account the previously observed variation in nanowire diameters (Figure 3.11), it is not easy to accurately correlate diameter change to the thickness of the antibody coating layer. The uniformity of antibody coverage was qualitatively assessed and it can be concluded that antibody coverage was relatively uniform over the entire wire, see Figure 3.22 (b).



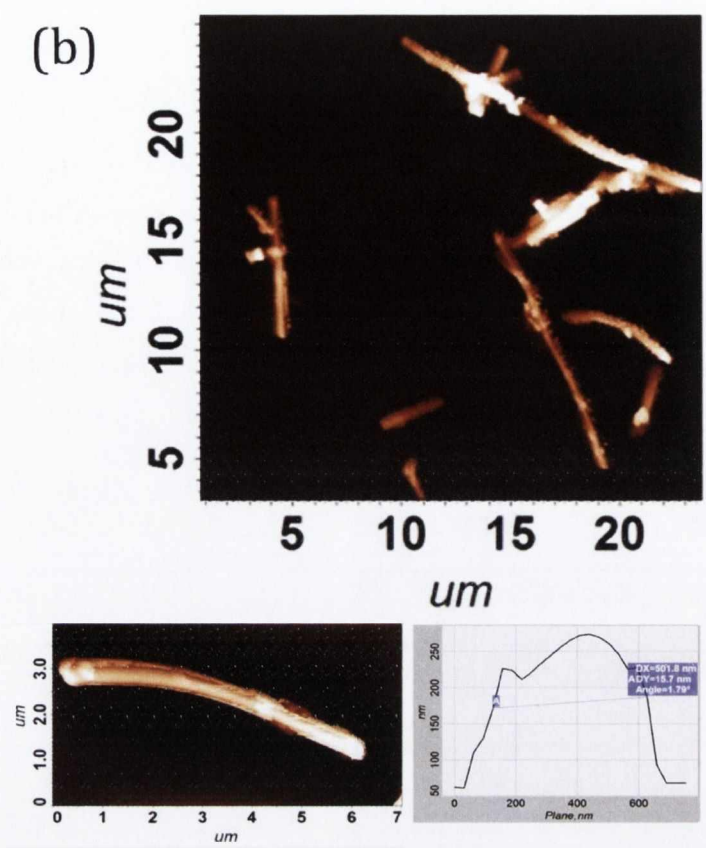


Figure 3.22 Assessment of nanowire functionalisation by atomic force microscopy. Non-functionalised (a) and functionalised (b) nanowires were imaged using atomic force microscopy. (a) The graph (lower right) shows the diameter of the non-functionalised nanowire to be 396.1nm. (b) Measurement of the nanowire after functionalisation with antibody shows an increase in the measured diameter to 501.8nm as illustrated in the graph (lower right). Both measurements refer to the nanowires presented in the upper right panels of the respective images.

3.2.7.3 Helium Ion Microscopy

Helium-ion microscopy (Figure 3.23) was used as a potential alternative to atomic force microscopy in assessing nanowire functionalisation. This time, a sample from the intermediate preparation step (APTES-coated nanowires) was included in the analysis, see figure 3.23 (b). Stock nanowires, see figure 3.23 (a), appeared smooth whilst both APTES-coated and fully functionalised nanowires, see figure 3.23 (b) & (c) respectively, showed increased surface roughness. The observed surface roughness was most pronounced in the antibody-functionalised sample, indicating the presence of antibodies on the nanowire surface.

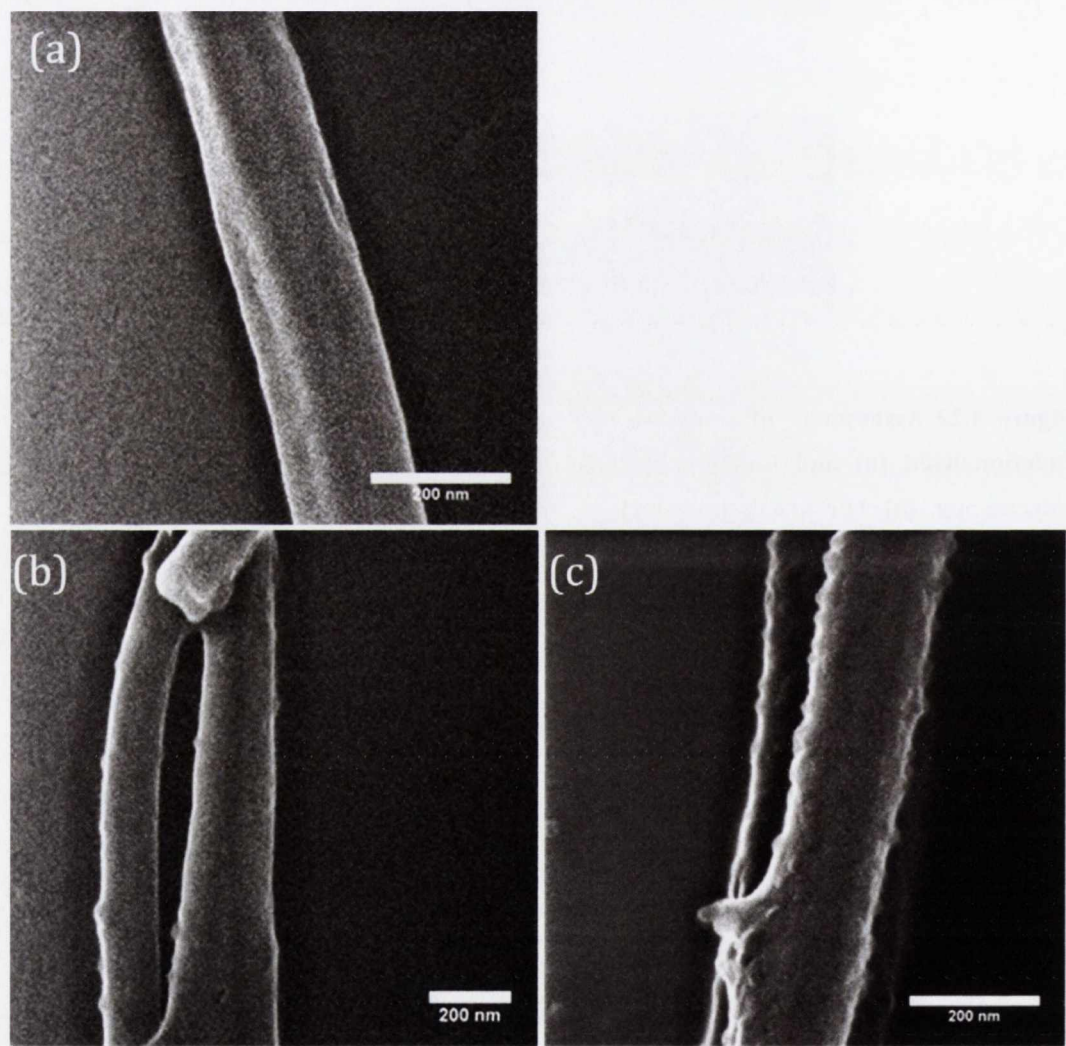


Figure 3.23 Assessment of nanowire functionalisation by Helium-ion microscopy. Helium-ion microscopy was used to examine non-functionalised nanowires (a), nanowires following APTES coating (b), and functionalised nanowires (c). Increased surface roughness is noticeable in both (b) and (c) and is most pronounced in the functionalised nanowire sample (c). Scale bar = 200 nm for all images.

3.2.7.4 Flow Cytometry

The functionalised nanowire population was examined using flow cytometric analysis (Figure 3.24). SSC thresholding was applied to the sample acquisition, and 10,000 events were captured for each sample. The samples were FITC-gated on the positive fluorescence signal from the purified antibody and then back-gated on to the nanowire population. This method enables to estimate the fluorescent nanowires population as a percentage of the overall nanowire population recorded within the sample. The results show that 43% of the fluorescent antibody-functionalised nanowires (Figure 3.24, bottom panel) emitted a signal in the FITC channel gate (P1), compared to 0.3% of the stock population (Figure 3.24, middle panel). The FITC-H mean was also larger in the functionalised nanowires (FITC-H Mean = 2057) compared to the non-functionalised nanowires (FITC-H Mean = 564). Since the preparation of antibody-coupled nanowire batches are different depending on the reactivity of the reagents, it is good practise to evaluate the functional yield of each individual batch prepared. This is common practice before the assay is validated and standardised. In this way, the concentration of nanowires used can be optimised to the “concentration of successfully functionalised nanowires” to be used in the assay.

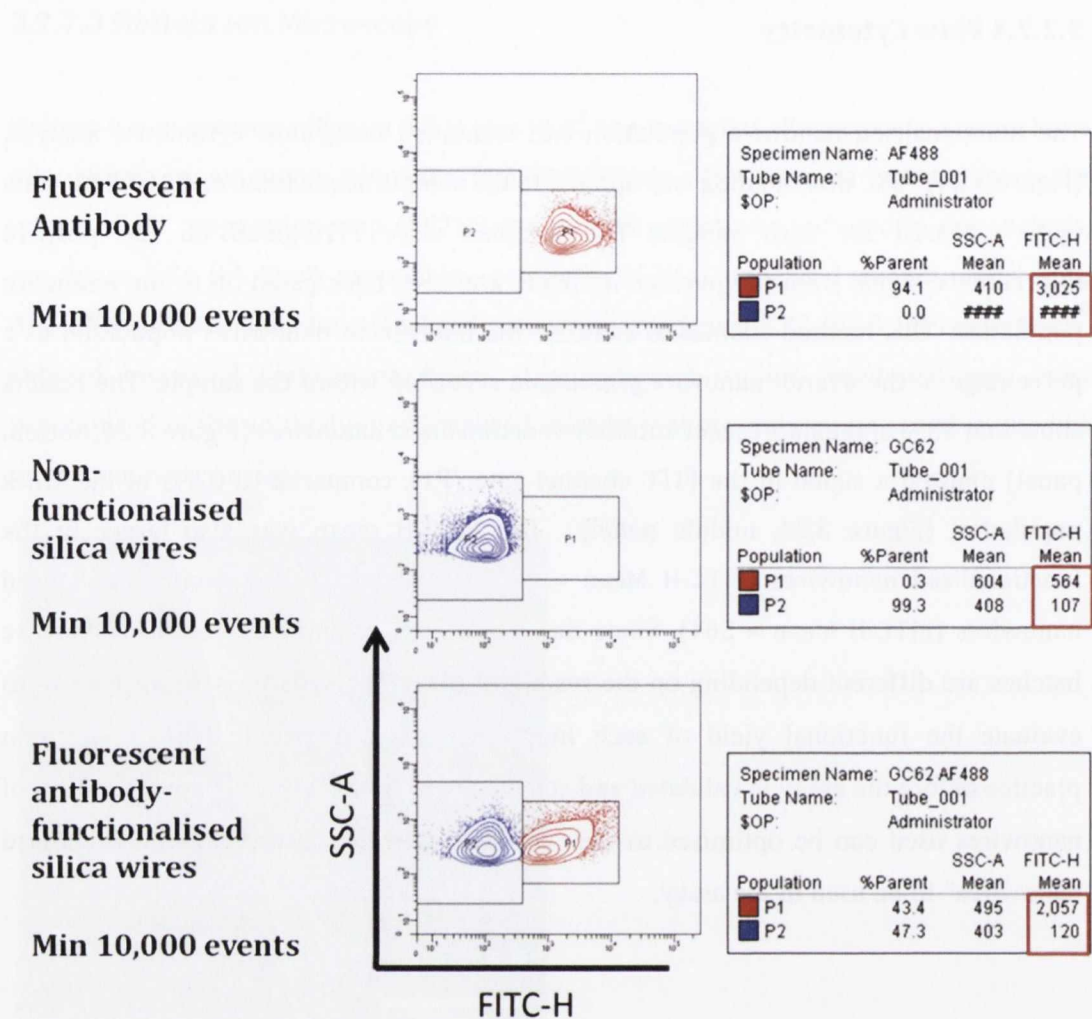


Figure 3.24 Assessment of nanowire functionalisation by flow cytometric analysis. Non-functionalised nanowires (middle panel) and fluorescent antibody-functionalised nanowires (bottom panel) were compared to fluorescent antibody (top panel) using a side scatter area (SSC-A) vs. FITC-H channel plot. The red box highlights mean FITC-H signal in each group for P1 (fluorescent) and P2 (non-fluorescent) populations.

3.2.8 Assessment of functionalised nanowire stability using flow cytometry

Following antibody conjugation to the nanowire surface, the nanoprobees were stored at 4 °C and assessed at regular intervals (weekly) for quality purposes. The stability of the antibodies on the nanoprobe surface of the functionalised nanowires (Figure 3.25 (b)) was assessed by comparing the sample to the anti-ErbB2-AF488 antibody positive control. The control antibody was visible in Q4 of the SSC-H vs FITC-H bi-exponential plot (Figure 3.25 (a)) and so it was postulated that any event found in Q4 constituted free antibody (i.e. antibody unbound from the nanowire surface). Nanoprobe stability assessment by flow cytometry demonstrated that antibodies remain conjugated to the nanoprobees for a prolonged period of time (over 28 days), as indicated by the lack of signal appearing in the Q4 quadrant of the functionalised nanowire population, see figure 3.25 (b). It is shown that <0.1% of the events measured in the functionalised nanowire population fell into Q4. This approach could not determine whether the biological activity of the antibody was still intact following tethering to the nanowire surface, however subsequent testing of the nanoprobees showed that the biological activity of the antibody did remain intact (see 3.2.10.1). Additionally, there is a possibility that the use of an unstable fluorophore would mean that the antibody coated nanowires would not be visible (even though the antibody may remain tethered to the nanowire and biologically active). This was not the case here however, as AF488 is considered a very stable fluorophore (see Appendix 7) and is ideally suited to applications requiring fluorescence integrity over prolonged periods of time, provided it is stored in the absence of light.

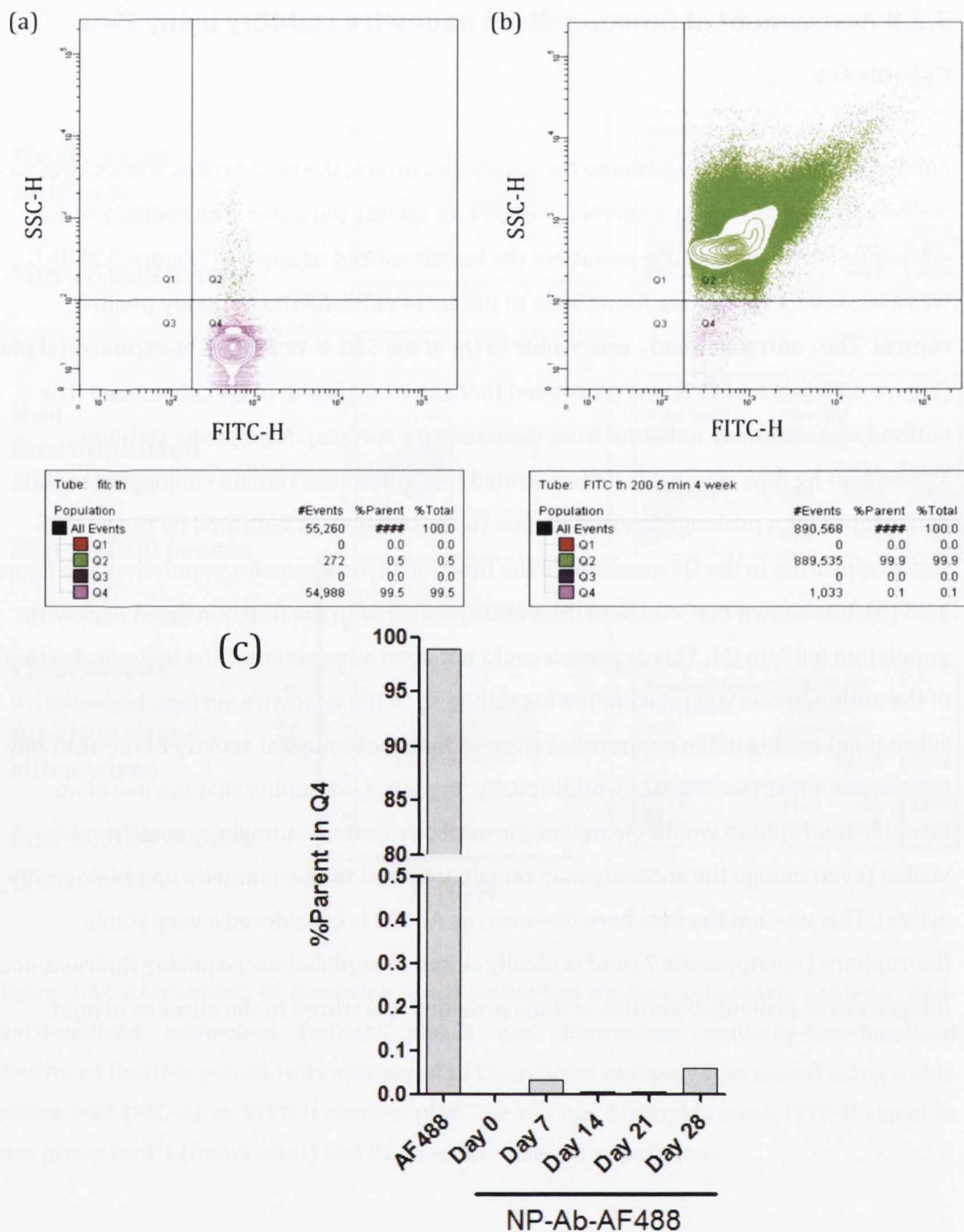


Figure 3.25 Assessment of functionalised nanowire stability using flow cytometry. (a) Anti-CD340-AlexaFluor488 is the positive control visible (99.5% population) in Q4 of the SSC-H vs FITC-H bi-exponential plot. (b) The nanoprobe functionalised with Anti-CD340-AlexaFluor488 show a similar fluorescent response to the fluorescent antibody alone but with a larger SSC-H signal. (c) The graph shows the percentage population in Q4 quadrant (indicating antibody dissociation from the nanowire) and shows less than 0.2% of the population in Q4 after 28 days of storage, compared to the fluorescent antibody alone as positive control.

This data indicates the presence of fluorescent antibody in the functionalised nanowire sample and supports the conclusion that the antibody is reliably immobilised on the nanowire. This method allows us to get a quantitative assessment of the percentage population of functionalised probe (by comparing the number of nanowires with a fluorescent signal vs. nanowires without a fluorescent signal) within a given preparation and allows the estimation of the batch-to-batch variability of antibody conjugation (by comparing the ratio of fluorescent to non-fluorescent populations between batches).

Validation of nanowire functionalisation with antibody is a crucial step in the development of a nanowire assay. Through the use of a number of complementary techniques (see section 2.4 for a synopsis of techniques used), the uniformity of antibody distribution was confirmed on the surface of the nanowire, and the proportion of the nanowire population which were successfully functionalised (in that they are visible by confocal and FACS) could be estimated (see section 2.2.8.4). Additionally, the stability of the nanowire-antibody conjugates was assessed by a custom developed flow cytometry approach (see section 3.2.8). The data indicates that the nanoprobe are stable for at least 28 days. Therefore, it is possible to use the nanowires prepared as described above for biological assays, confident that the antibody is reliably and consistently attached to the nanowire.

3.2.9 Characterisation of antibody-conjugated nanospheres

3.2.9.1 Fluorescent microscopy

Fluorescence-based microscopic analysis (Figure 3.26 (a), below) was carried out in order to visualise the fluorescent antibody on the surface of the nanosphere. The mean fluorescence intensity was recorded from the nanospheres (n=30) (excluding large aggregates) and compared to the background signal (the signal measured from nanosphere not conjugated with fluorescently-tagged antibodies), see figure 3.26 (b). The results demonstrate a change in the mean fluorescence intensity of nanospheres compared to background signal. This data supports the conclusion that the fluorescent antibody is present on the nanosphere. It should be mentioned that in all optical microscopes, including conventional confocal instruments, resolution (defined as the minimum separation between two points that results in a certain level of contrast between them) is restricted by the numerical aperture of optical components and by the wavelength of light, both incident (excitation) and detected (emission) with shorter wavelength radiation allowing better resolution.

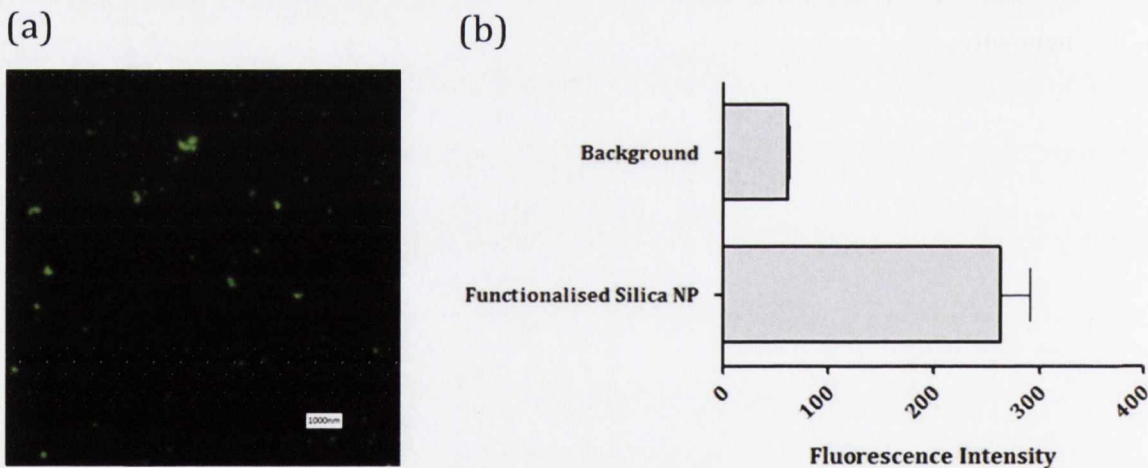


Figure 3.26 Fluorescent microscopic analysis of nanospheres. Confirmation of antibody attachment to nanospheres was demonstrated by fluorescent microscopy. Fluorescence intensity from antibody-conjugated nanospheres (a) was measured and plotted against the background (b). The data is presented as mean values $\pm$ standard deviation from the mean. Scale bar = 1 μ m.

3.2.9.2 Nanoparticle Tracking Analysis

Functionalised nanospheres were compared to stock nanospheres via Nanosight tracking analysis NTA (Figure 3.27). Compared to the non-functionalised nanospheres in figure 3.27 (a), results show that aggregation occurs in the functionalised sample (Figure 3.27 (b)), indicated by secondary size peaks between 280 – 500 nm. The mean and mode nanosphere sizes were calculated using the NTA software (Table 3.2). The results demonstrate that while the mean nanosphere size was 254.7 nm, the mode nanosphere size was 142.0 nm. The concentration of the dispersed particles was estimated by NTA software and provided a basis for determining the quantity of functionalised nanospheres to be used in subsequent assays. The concentration of nanoparticles used in the assay was calculated through comparison to theoretical reactive surface area of the bead assay (i.e. theoretical surface area of bead X number of beads used per assay sample). Various concentrations of capture antibody-nanoparticles [Range: 1×10^4 - 1×10^6 NW/ml], detection antibody and fluorescent secondary antibody [Range 1 - 10 $\mu\text{g/ml}$] were trialled during the optimisation process in order to obtain the best signal-to-noise ratio for assay analysis.

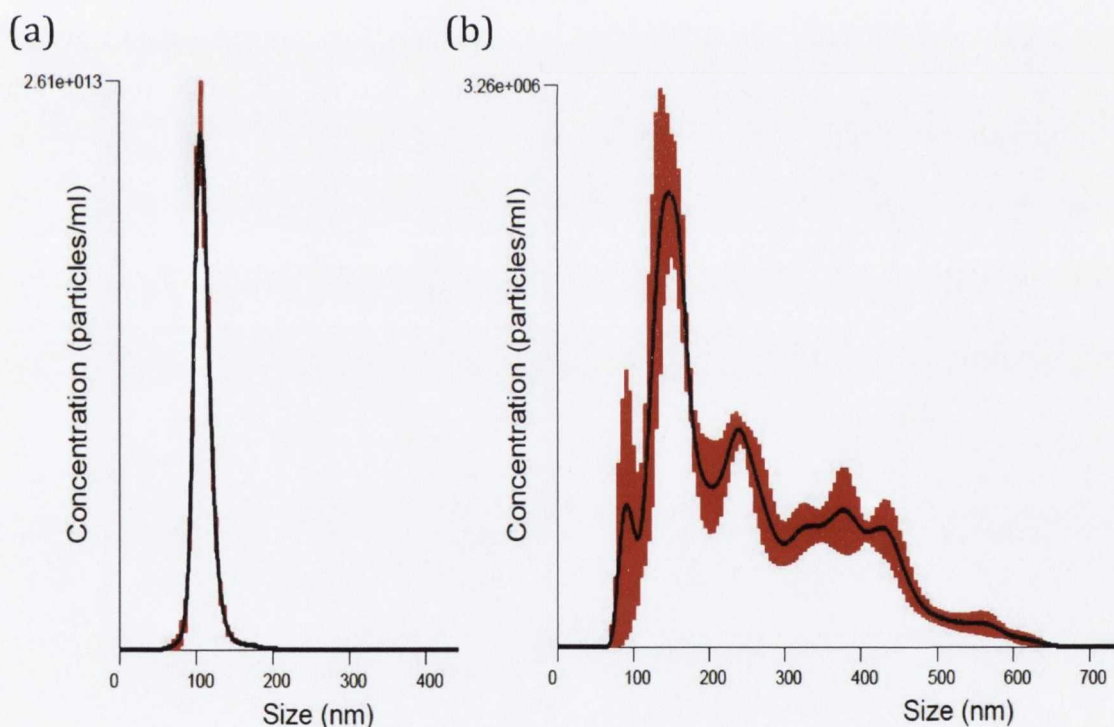


Figure 3.27 Size analysis of nanospheres using NTA on the Nanosight NS500. Non-functionalised nanospheres (a) and functionalised nanospheres (b) were measured six times and sphere size distribution was plotted using the NS500 software. Results displayed as mean (black line) +/- std. error of the mean (vertical red lines).

Table 3.2 Mean and mode diameter of nanospheres as determined by NTA.

	Stock Silica Nanospheres	Functionalised Silica Nanospheres
Mean Size	110.0 ± 0.4 nm	254.7 ± 10.8 nm
Mode Size	101.1 ± 1.1 nm	142.0 ± 15.1 nm

3.2.9.3 Flow cytometry

Non-functionalised and functionalised nanospheres (otherwise known as non-labelled and labelled, respectively) were examined by flow cytometry (Figure 3.28). FITC threshold was applied to the sample acquisition and each sample was run until 10,000 events were captured or 5 min elapsed, whichever came first. Twenty events were recorded in the non-functionalised nanosphere sample (Figure 3.28 (a), below) after 5 min acquisition time, meaning that there was negligible background fluorescent signal present. A total of 10,000 events were recorded in the functionalised nanosphere sample, with the majority (99%) recorded in the FITC channel gate (P1) as a clear fluorescent peak (Figure 3.28 (b), below). This data indicates the presence of fluorescent antibody in the functionalised nanosphere sample and suggests that the antibody is coupled to the nanosphere.

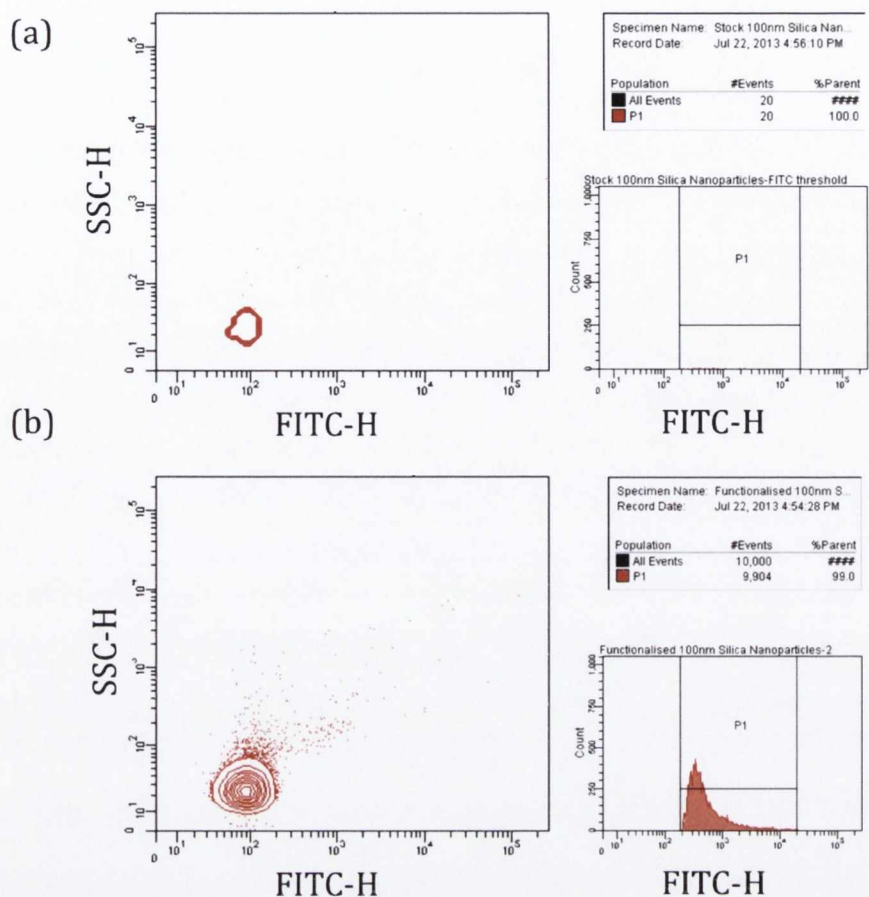


Figure 3.28 Flow cytometric analysis of nanospheres. Stock nanospheres (a) and functionalised nanospheres (b) were analysed using flow cytometry. (a) For stock nanospheres, a very low number of events (20 events) was recordable when the FITC channel (Emission filter 530/30) threshold was applied. (b) A significant number of functionalised nanospheres (10,000 events) were visible under the same FITC threshold, indicating the presence of fluorescent antibody. This is illustrated in both the scatter plot (left) and the FITC-H histogram (right).

3.2.9.4 Spectroscopy

Functionalised nanospheres and non-functionalised nanospheres were also examined spectroscopically, compared to purified fluorescent antibody (IgG-AlexaFluor488). The absorbance values for each sample in the wavelength interval between 300 nm and 800 nm was recorded at 10 nm intervals (Figure 3.29). An absorbance peak at 495 nm was observed both in the purified fluorescent antibody and in the functionalised nanosphere samples. This is indicative of the fact that the fluorescent antibody has remained on the nanospheres following the functionalisation and wash processes. The absorbance peak is not registered for the non-functionalised nanosphere sample, indicating that the absorbance peak was not attributable to the nanospheres themselves. The apparent absorbance of the stock silica particles as determined by the spectrometer is more likely to arise from dispersion of the incident light related to the particle size, rather than from direct absorbance. This hypothesis (based on the assumption of Mie scattering) is supported by the observation that the functionalised NPs (containing larger particles in the form of aggregates) have a higher dispersion at longer wavelengths than the stock non-functionalised NPs.

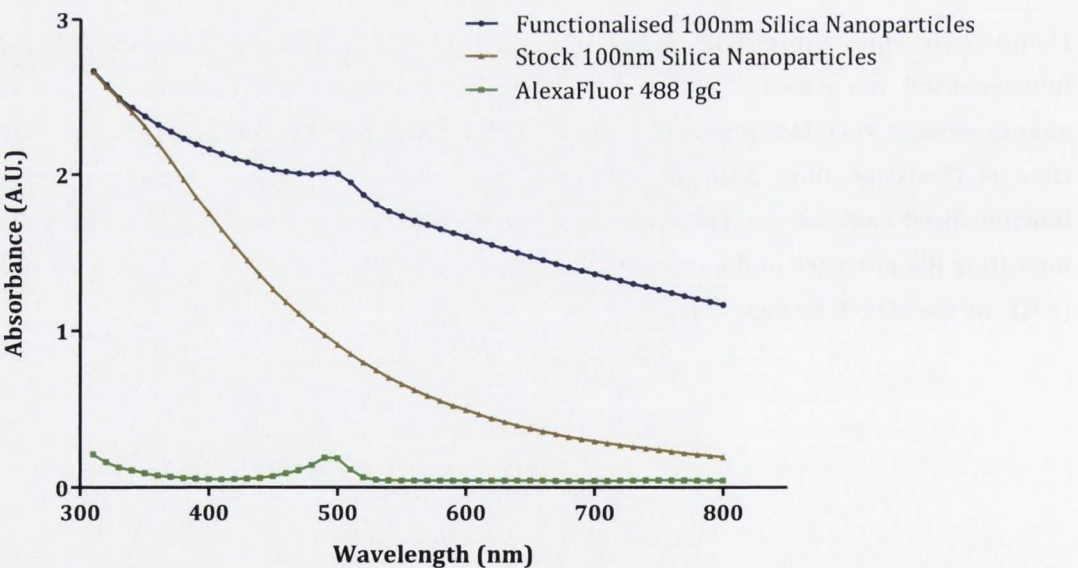


Figure 3.29 Spectroscopic analysis of nanosphere functionalisation. The spectral absorbance profiles of stock nanospheres (brown line), functionalised nanospheres (blue line) and purified fluorescent antibody (green line) are shown. Absorbance peaks can be observed both in the fluorescent antibody and the functionalised nanosphere samples at 495nm. A.U.=Arbitrary Units.

3.2.9.5 Gel Electrophoresis

Gel electrophoresis of functionalised and non-functionalised nanospheres was performed (Figure 3.30). The results show that while the non-functionalised nanospheres migrated toward the cathode, the functionalised nanospheres remained at the loading point. It is possible that either the functionalised nanospheres underwent a change in charge or size or both during the functionalisation process and this caused the nanospheres to remain at the loading point. This result suggests that the antibody is present on the nanosphere surface.

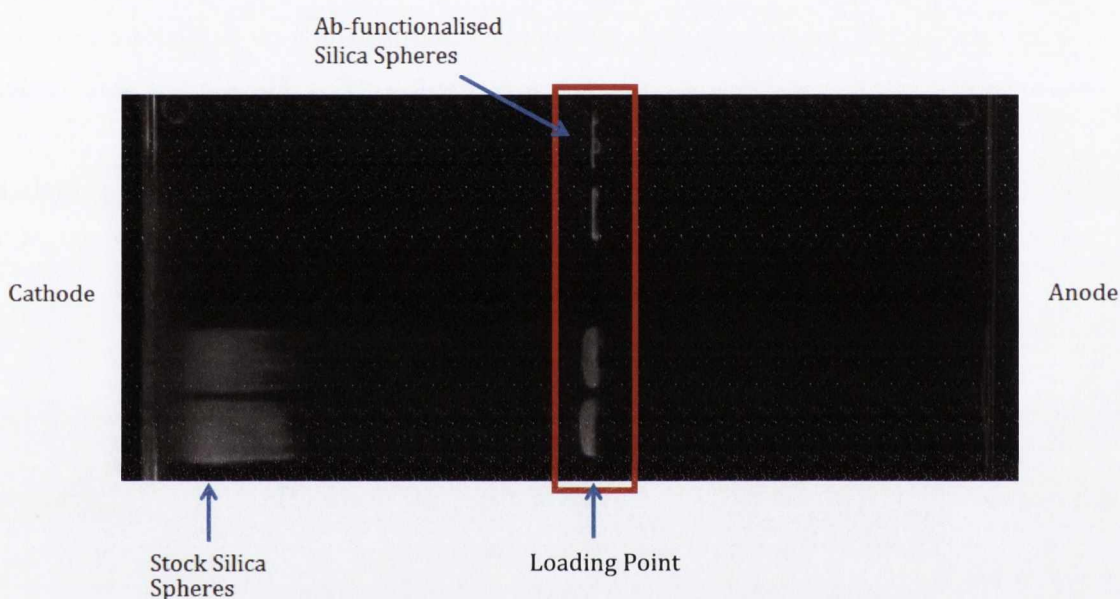


Figure 3.30 Gel electrophoresis of silica nanospheres. During the electrophoretic separation, non-functionalised (stock) silica nanospheres migrated towards the cathode from their loading point in the gel. Functionalised silica nanospheres remained in their loading point throughout the electrophoresis process.

These data taken together indicate that the fluorescent antibodies were attached to the surface of the nanomaterials. The combination of various analytical and imaging approaches (see section 2.4 for synopsis of techniques used) allows us to get a quantitative and qualitative assessment of the quality of the functionalised nanoprobe within a given preparation. This part of work highlights the similarities and differences in analytic approaches to determining antibody conjugation with nanowires and nanospheres. For example, fluorescent confocal microscopy and flow cytometry can be used to assess both types of nanomaterials, whereas gel electrophoresis and Nanosight Tracking Analysis is only suitable for the analysis of nanospheres. Our observations underline the need to explore multiple analytic options, depending on the geometry of the material under investigation.

3.2.10 Proof-of-concept testing of the developed nanowire platform

3.2.10.1 Detection of sErbB2 using the developed nanowire platform

A proof-of-concept detection assay using functionalised nanowires was developed. This “sandwich assay” was implemented and involved functionalisation of nanowires with a non-fluorescent anti-ErbB2 capture antibody (Trastuzumab), incubating the nanowires with either sErbB2 or BSA solution, and finally adding a fluorescent anti-ErbB2 detection antibody (anti-CD340-AlexaFluor488). Each of the samples was examined using fluorescent confocal microscopy and flow cytometry (Figure 3.31). The results demonstrate sErbB2 capture on the nanowire which is confirmed by a fluorescent signal from the detection antibody (Figure 3.31, upper panel) by confocal microscopy and flow cytometry. In the presence of a non-specific target such as BSA, the analyte is not captured by the nanowire and there is no signal detection observed using confocal microscopy. Analysis of the BSA-incubated nanowires using flow cytometry reveals a weak fluorescent signal from the nanowire population which can be interpreted as a background signal resulting from non-specific binding effects.

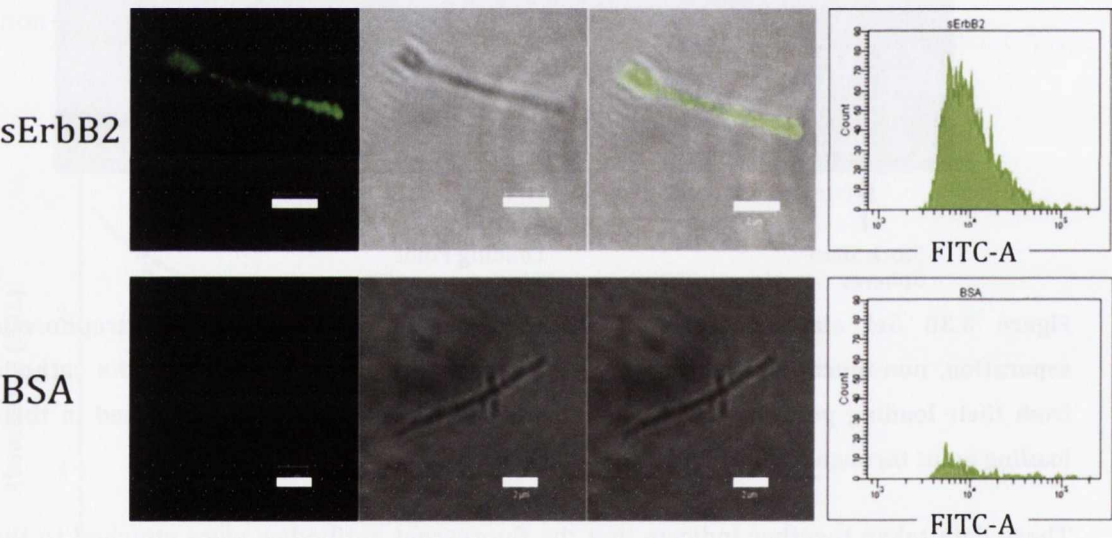


Figure 3.31 Detection of sErbB2 in a single analyte system using confocal microscopy and flow cytometry. In the presence of sErbB2, the nanowire captures the analyte and detection is achieved by the addition of a fluorescent anti-ErbB2 antibody (upper panel). The fluorescent signal can be seen using confocal microscopy (left) and flow cytometry (right). In the presence of BSA, no signal is detected using confocal microscopy and a low fluorescence signal is seen using flow cytometric analysis (lower panel). Scale bar =2 μm .

These results demonstrate the nanowire assay as capable of differentially detecting an analyte (sErbB2) and uses fluorescence from a tagged antibody to confirm presence of sErbB2 by two separate approaches.

3.2.11 Advanced assay development

3.2.11.1 Detection of an sErbB2 concentration range in a single analyte model

The FITC-tagged anti-ErbB2 monoclonal antibody (mAb) (BMS120FI) as capture antibody and anti-ErbB2 polyclonal antibody (pAb) (AF1129) as detection antibody were selected by collaborators Progenika Pharma as the best antibody pairs for the detection of sErbB2 and so these antibodies were substituted into the nanowire platform in place of Trastuzumab and anti-CD340-AlexaFluor488, respectively. Since the new detection antibody was not fluorescently tagged, a PE-tagged secondary antibody was used to detect the presence of anti-ErbB2 pAb (AF1129) on the nanowire. The nanowire assay was tested to see if detection of a concentration range of sErbB2 in a single analyte model (sErbB2 in PBS solution) was possible. The non-specific antibody-conjugated (IgG) nanowires were used to test whether the presence of a non-specific antibody on the nanowire would cause non-specific binding of sErbB2 to the nanowire probe (Table 3.3). The appropriate FACS controls were recorded and signal compensation was carried out (see Materials and Methods section 2.2.9.3 for controls and gating strategy) before data acquisition. The PE-H Median values from sample were recorded. Figure 3.32 (right graph) shows that the recorded PE-H Median values increased with increasing concentration of sErbB2 in solution. In addition, the nanowire sample functionalised with a non-specific antibody did not have an optical signal in the PE channel at 0 ng/ml and 1000 ng/ml sErbB2.

Table 3.3 Assay procedure for detection of an sErbB2 concentration range using anti-ErbB2 nanoprobe.

Protocol steps	Nanoprobes blocked with Casein diluent	sErbB2 added to nanoprobes	Wash	Addition of anti-ErbB2 Detection Ab	Secondary Ab PE.	Wash
Time	2hr	2hr	1hr	2hr (Both Abs added at same time)		1hr
Detail	Anti-ErbB2 Nanowire (1x10 ⁵ NW/ml)	1000.0 ng/ml	PBS+ Tween 0.02% two times.	6.7 µg/ml	5.0 µg/ml	PBS+ Tween 0.02% three times.
		250.0 ng/ml				
		62.5 ng/ml				
		0 ng/ml				
	IgG Nanowire (1x10 ⁵ NW/ml)	1000.0 ng/ml				
		0 ng/ml				

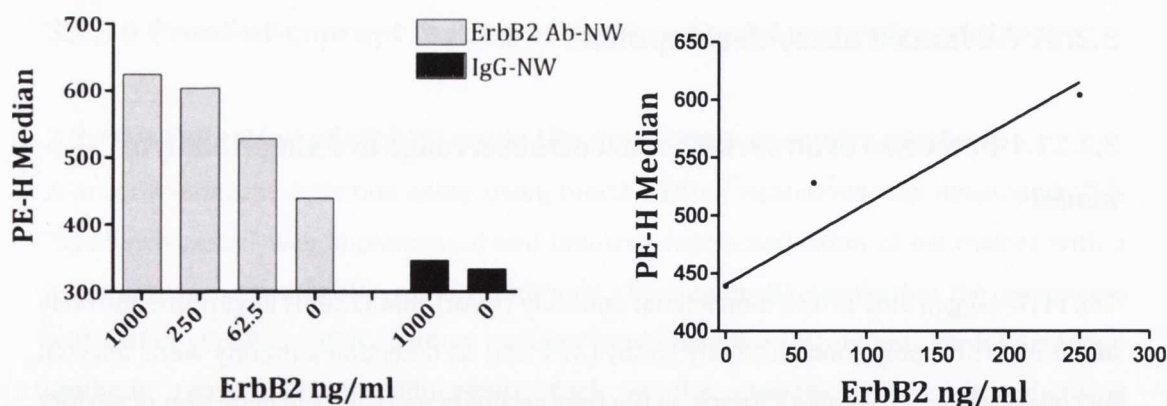


Figure 3.32 Detection of ErbB2 concentration range in single analyte model. Right graph: PE-H Median values correlate positively with a concentration range of ErbB2. The PE-signal response in the IgG-nanowire samples (dark grey bars) was lower than those of the anti-ErbB2-nanowire samples (light grey bars). **Left graph:** The first three data points for the ErbB2 Ab-MW were re-plotted on a dot plot to assess whether the response curve was linear. Using a linear curve fit, it appears that the response curve is not especially linear over the concentration range.

The data indicates that the PE-signal associated with the anti-ErbB2 nanowires increases in the presence of increasing sErbB2 concentration and supports the suggestion that the increased PE-signal is as a result of the presence of sErbB2 on the nanowires. Conversely, the PE-signal recorded from the non-specific antibody (IgG)-conjugated nanowire population was low regardless of the sErbB2 concentration in solution, indicating that the specific anti-ErbB2 capture antibody was required for sErbB2 capture. The first three data points for the ErbB2 Ab-MW were re-plotted on a dot plot to assess whether the response curve was linear (Figure 3.32, left graph). Using a linear curve fit, it appears that the response curve is not especially linear over the concentration range

3.2.11.2 Detection of spiked sErbB2 in blood plasma and comparison of nanosphere and nanowire assays

Spiked sErbB2 in reconstituted human blood plasma was used to test whether the anti-ErbB2 nanowire platform could detect sErbB2 in a complex biological sample. In addition, the performance of anti-ErbB2 nanowires were compared to anti-ErbB2 nanospheres functionalised with the same antibodies (Table 3.4). A 10% Universal Casein Diluent in PBS was used as a blocking buffer and a PBS wash buffer containing 0.02% Tween 20 detergent provided satisfactory results in terms of low background non-specific binding. Using wash buffers with a higher Tween 20 content resulted in the catastrophic loss of

nanoprobe during the wash steps of the assay, most likely because use of Tween increased the stability of the nanoprobe in solution and preventing them from being separated from the dispersion.

Table 3.4 Assay procedure for detection of spiked sErbB2 using anti-ErbB2 nanowires and nanospheres.

Protocol steps	Nanoprobe blocked with Casein diluent	sErbB2 added to nanoprobe	Wash	Addition of anti-ErbB2 Detection Ab	Secondary Ab PE.	Wash
Time	2hr	2hr	1hr	2hr		1hr
Detail	Nanowires or nanospheres (1x10 ⁵ NW/ml) incubated with 10% Universal Casein Diluent	100.0 ng/ml	PBS+ Tween 0.02% two times.	6.7 µg/ml	5.0 µg/ml	PBS+ Tween 0.02% three times.
		50.0 ng/ml				
		20.0 ng/ml				
		0 ng/ml				

Following the assay procedure detailed in table 3.4, flow cytometric analysis of the nanosphere, see figure 3.33 (a), below, and nanowire, see figure 3.33 (b) below, assays were carried out. The data is expressed as the ratio of nanoprobe with a concurrent PE-signal (P1) to nanoprobe without a PE-signal (P2), within each sample. The results show that an increase in PE-signal correlated with an increase in spiked sErbB2 in the nanowire system. This was not the case for the nanosphere assay, which showed a higher P1:P2 signal at 50 ng/ml compared to the 100 ng/ml sample. However, the nanosphere assay showed an increase in PE-signal in proportion to the increase in sErbB2 concentration for the 0 nm/ml, 25 ng/ml and 50 ng/ml sErbB2 samples. The mean P1:P2 values gained from the nanowire assay increased proportionally with increasing sErbB2 concentration across the entire range tested.

These results support the suggestion that sErbB2 is detectable in a complex biological sample using the introduced nanowire assay. Although the nanosphere assay showed a larger relative signal difference between 25 ng/ml sErbB2 and 0 ng/ml sErbB2 samples compared to the nanowire assay, the latter provided a more linear response to the increasing sErbB2 concentrations. It is important to consider that while the nanosphere assay outperformed the nanowire assay over the initial 0-25 ng/ml range, the

concentrations of sErbB2 in a clinical setting stretch beyond this concentration point. In clinical practice, a cut-off threshold of 15 ng/ml sErbB2 is used, above which point the patient is considered to have elevated levels of sErbB2. Indeed the concentration of sErbB2 measured in biological fluids can extend past 100 ng/ml sErbB2. For this reason, the linearity of the response of the nanowire assay was seen as favourable, as it stretched over a much wider range (covering a larger portion of the clinically relevant assay window) than the nanosphere assay.

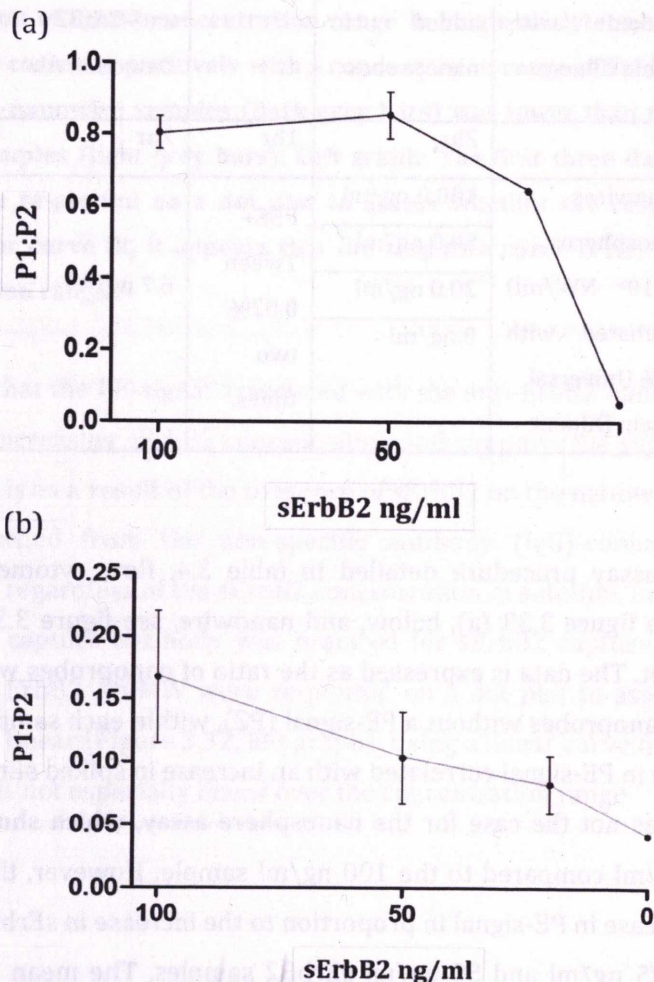


Figure 3.33 Detection of spiked sErbB2 in reconstituted plasma using the nanosphere (a) and nanowire (b) assay platforms. Data expressed as the ratio of wires with a concurrent PE signal (P1) vs wires without a concurrent PE signal (P2). Data is presented as mean $\pm$ std. deviation.

It was identified during the nanoprobe assay development that the fluorophore (FITC) for use in tagging the nanoprobe for cytometric detection may be suboptimal, affecting the sensitivity and reliability of the results. Thus, a comparison of fluorophores was conducted to identify a suitable replacement.

3.2.11.3 Comparison of fluorophores

A comparison of fluorophores was conducted in order to establish whether FITC was a suitable fluorophore for use in the development of a high-sensitivity nanowire assay and whether other fluorophores might provide better alternatives to FITC use. Three fluorophore-linked antibodies were compared based on their similar spectral emission wavelengths (i.e. $\sim 520\text{nm}$): FITC-antibody (eBioscience, Germany), AlexaFluor488-antibody (Biolegend, USA) and CF488-antibody (Sigma-Aldrich, USA). Each antibody was firstly conjugated to glass (silica substrate) and examined by microscopy using fluorescence-based analysis (Figure 3.34). The results show that glass with FITC-antibody conjugated to the surface had a low mean fluorescence intensity compared to glass conjugated with either CF488-antibody or AlexaFluor488-antibody. The fluorescent intensities of CF488 and AlexaFluor488 were similar.

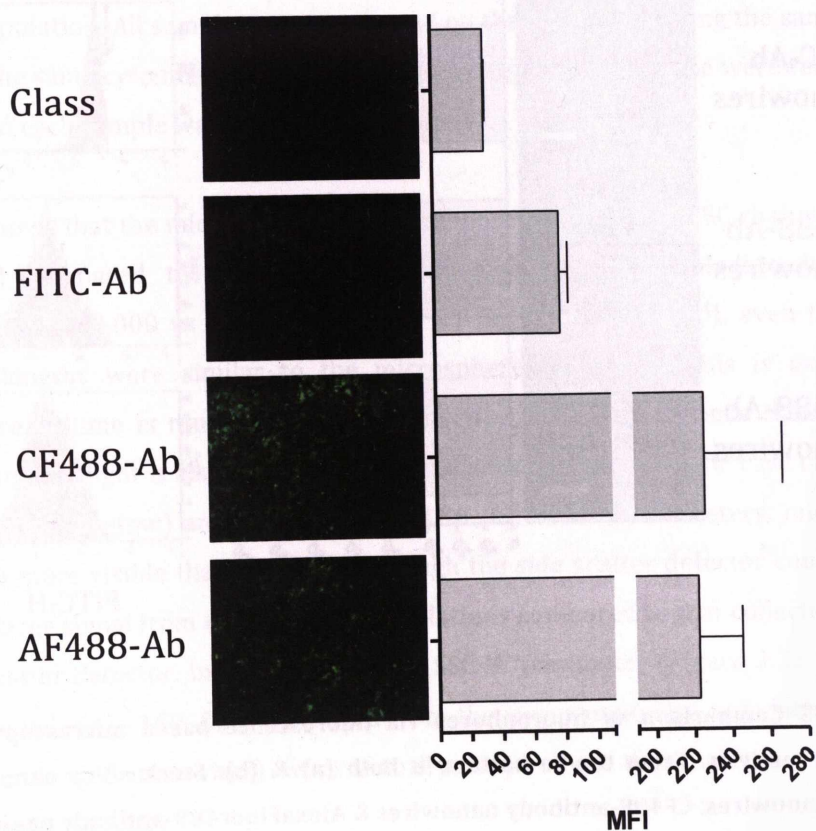


Figure 3.34 Comparison of fluorophores on glass via fluorescence-based analysis. Microscopic images from top to bottom: Glass substrate alone, FITC-antibody (FITC-Ab), CF488-antibody (CF488-Ab) & AlexaFluor488-antibody (AF488-Ab). MFI: Mean Fluorescence Intensity. Data in graph presented as mean $\pm$ std. deviation.

Each antibody was then conjugated to silica nanowires and examined by fluorescent microscopy (Figure 3.35 (a) below). Each antibody-conjugated nanowire sample was also

analysed using flow cytometry (Figure 3.35 (b) below) with FITC threshold applied. The results show that FITC-antibody conjugated nanowires showed lower fluorescence intensity compared to either CF488- or AlexaFluor488-conjugated nanowires. The MFI measured for FITC-antibody nanowires was about 5 times less than that of CF488-antibody or Alexafluor488-antibody nanowires. Flow cytometric data, see figure 3.35 (b) supported the microscopic analysis data. Measurement of the geometric mean values recorded by the flow cytometer show that FITC-antibody nanowires are less intense in the FITC-H channel.

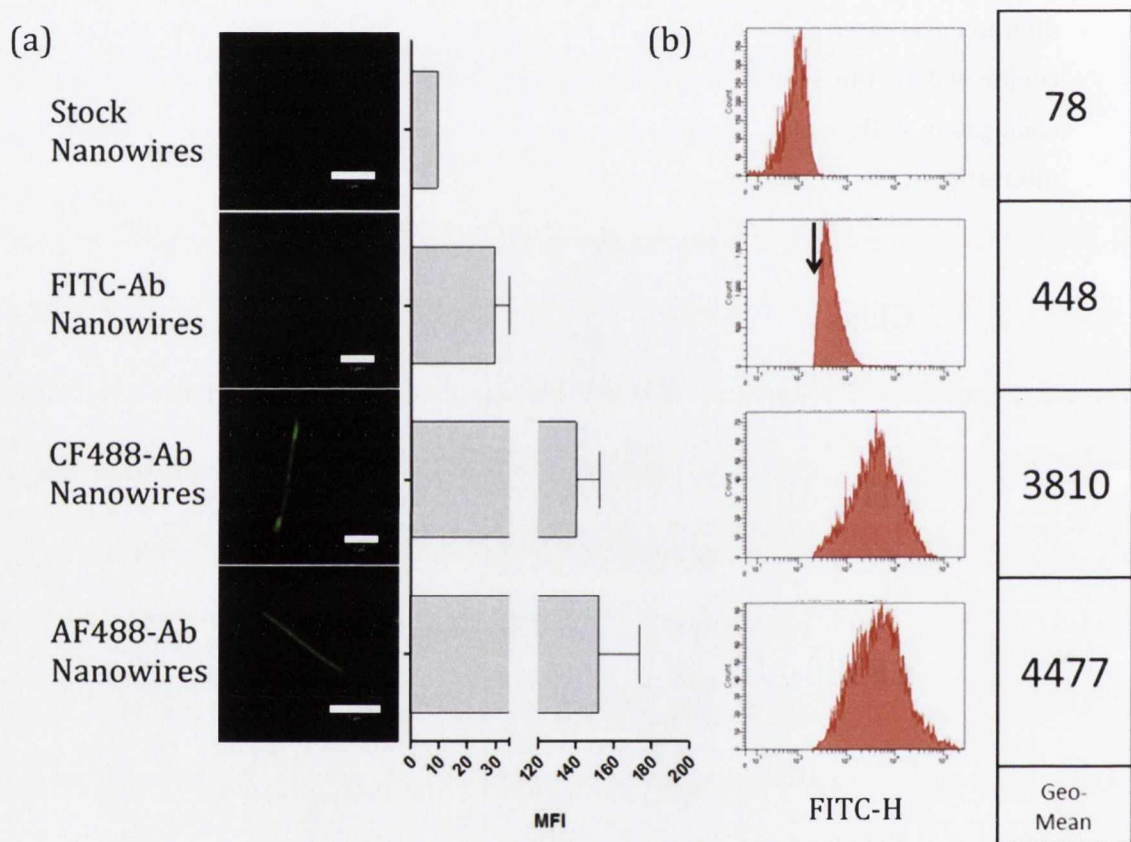


Figure 3.35 Comparison of fluorophores via fluorescence based microscopy and flow cytometric analysis. From top to bottom in both (a) & (b): Stock silica nanowire, FITC-antibody nanowires, CF488-antibody nanowires & AlexaFluor488-antibody nanowires. The data represented in the bar chart (a) is based on measurements taken from 30 nanowires per group and calculated to correct for background fluorescent signal. MFI: Mean Fluorescence Intensity. Scale bar = 2 μm.

Both sets of results results show that the FITC-antibody has suboptimal spectral properties compared to either CF488- or AlexaFluor488- conjugated antibodies, and thus

FITC fluorophore was not suitable for the nanowire assay. CF488-antibody was selected for use in subsequent nanoprobe platforms.

3.2.12 Analysis of nanowire signatures using flow cytometry

3.2.12.1 Comparison of scatter properties of nanowires and microspheres using flow cytometry

Ag nanowires (AgNWs) and microspheres of various sizes (size distributions for each nanowire and microsphere group were determined from the SEM measurements presented in section 3.2.5) were analysed in the flow cytometer and the median values of Forward Scatter Area, Height and Width (FSC-A, FSC-H & FSC-W, respectively) as well as Side Scatter Area, Height and Width (SSC-A, SSC-H & SSC-W, respectively) were calculated for each population. All samples were measured on the same day during the same session and using the same cytometer settings. 100,000 events for each sample were recorded for analysis and each sample was measured in triplicate.

The data shows that the microspheres are much more visible in the FSC channels (Figure 3.36 (a-c)) compared to the nanowires (for example; FSC-A Median for 15 μm microsphere = 260,000 vs FSC-A Median for 21 μm nanowire = 20), even though the nanowire lengths were similar to the microsphere diameters. This is expected, as microsphere volume is much larger than nanowire volume and hence their ability to disperse incident light is also increased. The data also shows that in the case of the SSC-A (Figure 3.36 (d), below) and SSC-H (Figure 3.36 (e), below) parameters, microspheres were much more visible than nanowires, though the side scatter detector could collect a relatively large signal from the nanowire populations compared to that collected from the forward scatter detector. Interestingly, in the SSC-W parameter (Figure 3.36 (f), below), the median values ($\sim 150,000$) measured from the larger nanowire populations (21 μm and 26 μm length nanowires) were actually larger than those of the microsphere populations ($\sim 100,000$).

This data suggests that SSC-W might be the most preferable parameter with which to view nanowire populations and that the nanowire signal magnitude in the SSC-W parameter is similar to that of similarly sized microspheres. SSC-W may be a key parameter to consider in order to better allow the integration of nanowires into flow cytometric platforms, as shown in figure 3.36 (f), below.

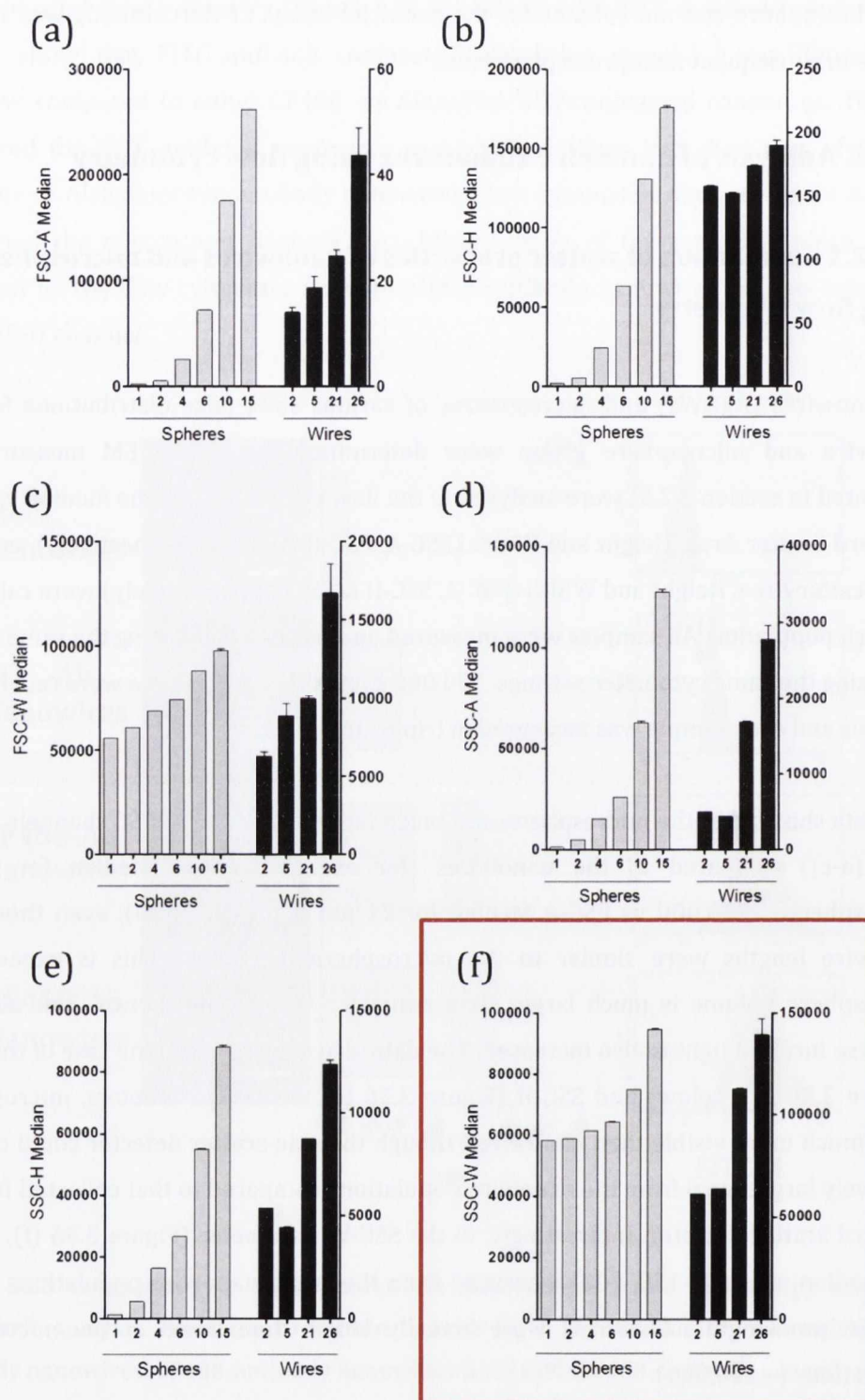


Figure 3.36 Flow cytometric comparison of microspheres and nanowires using forward-scatter (FSC) and side-scatter (SSC) parameters. Microsphere (labelled as spheres) and nanowire (labelled as wires) batches were analysed using the FSC-A, FSC-H, FSC-W, SSC-A, SSC-H and SSC-W parameters (100,000 events recorded per sample). Median values for each parameter are the representative data displayed (a-f). The SSC-W parameter is the only parameter where nanowires show a larger magnitude optical signal than microspheres. (Please note differences in double Y-axes, maximum values vary)

3.2.12.2 Comparison of SSC-W response of AgNWs and microspheres using flow cytometry

From initial comparative observations, it was noticed that SSC-W was the only parameter in which the nanowires and microspheres of comparable dimensions had similar signal outputs. For all other parameters, microspheres yielded a much larger magnitude signal than wires. The SSC-W data was further visualised using FlowJo software (USA), and the results (Figure 3.37) are displayed below. The data reveals that in SSC-W histogram plot, nanowires (b) yield a larger SSC-W response than microspheres (a). The 26 μm long nanowires (median measurement as measured by SEM) show a major population peak above 250,000 (250K) on the SSC-W plot. This observation suggests that SSC-W could be used to measure length-dependent optical signals from nanowires.

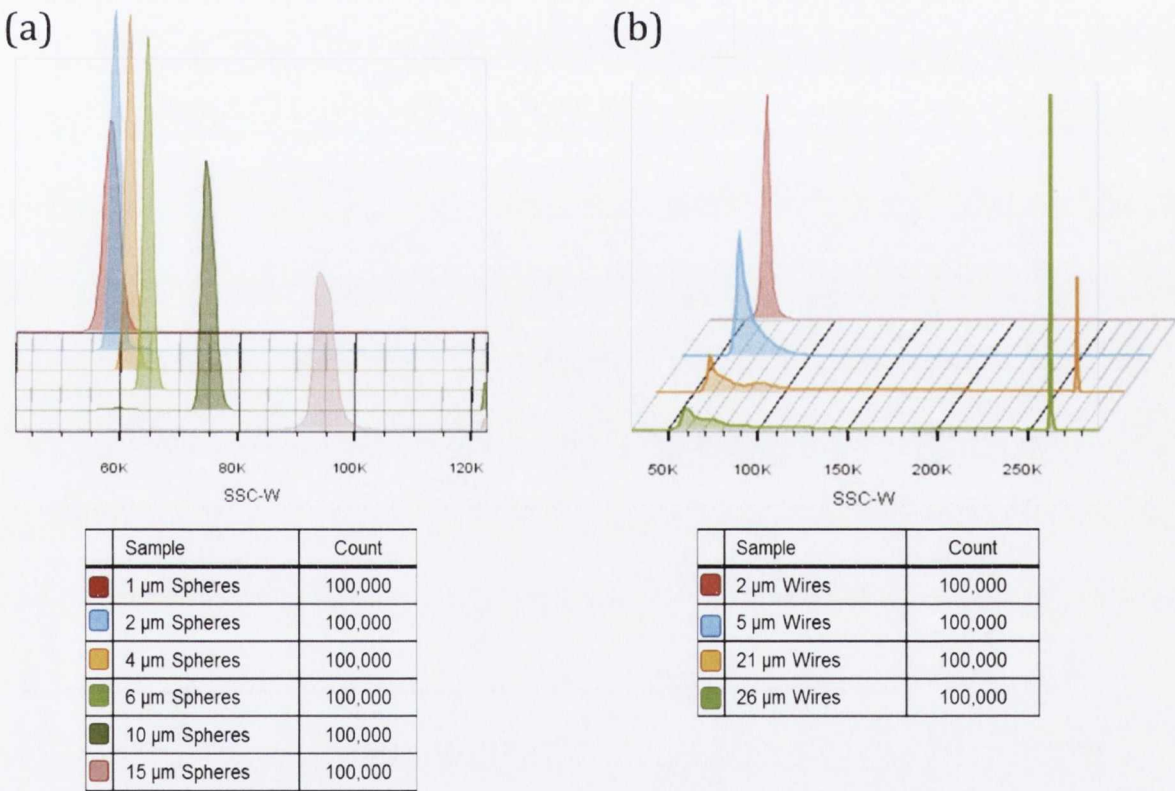


Figure 3.37 Comparison of SSC-W response between microspheres and nanowires. Flow cytometric analysis of microspheres (a) and nanowires (b) was conducted using the SSC-W parameter. A chart displaying the colour representing each sample is displayed directly beneath each histogram. 100,000 events were collected for each sample. Data was visualised using FlowJo flow cytometry software. [K = *1000]. Visualisation of the microspheres in the SSC-W plot shows that the SSC-W signal is proportional to the microsphere size. Visualisation of the nanowire populations shows that the SSC-W signal is also proportional to the nanowire size, with a higher proportion of the longer nanowires recorded with a large SSC-W signal.

3.2.12.3 Gating strategy for analysis of length-associated optical response in nanowires

A custom gating strategy was developed to analyse the length-associated optical response in of nanowire populations as recorded by the SSC detector. The novel gating strategy built on the comparative observations reported in the previous section, which found that SSC-W was the only parameter where spheres and nanowires of similar dimensions had similar signal outputs (see figure 3.36 (f) in section 3.2.12.1). This gating strategy was built around the analog “pulse” recorded by the SSC detector (before the signal is digitised and added to the histogram plot seen in Figure 3.39). Each detected light pulse has an intensity, duration and integral (SSC-H, SSC-W and SSC-A, respectively) and is illustrated in Figure 3.38 below. It is hypothesized that nanowires will have a proportionally larger SSC width-to-height ratio due to their geometry, compared with spheres in which the geometry is the same size in all directions.

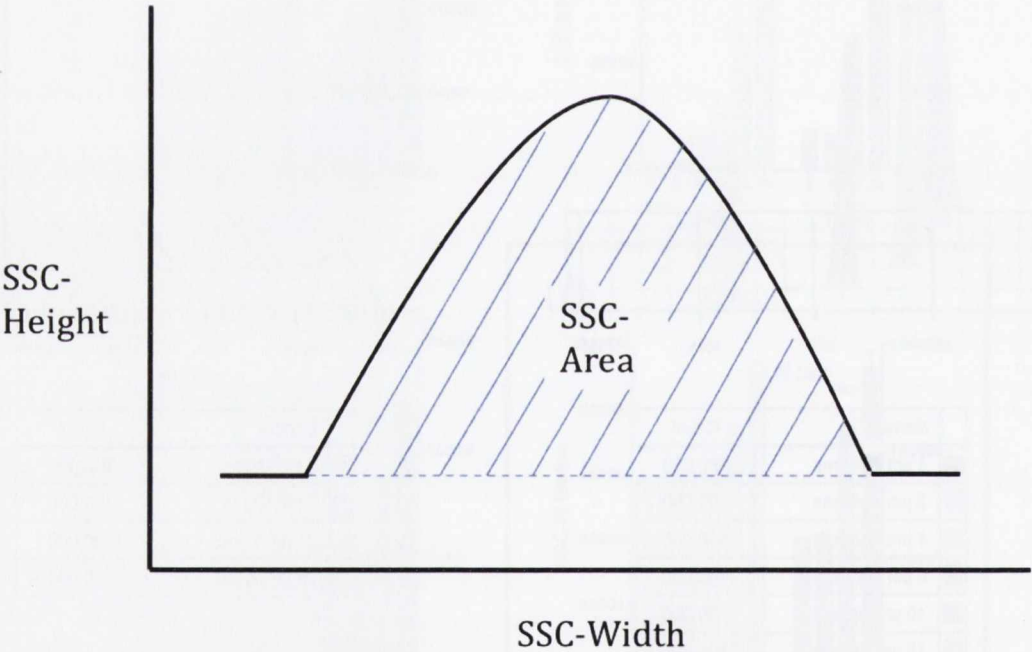


Figure 3.38. Schematic of light pulse. Schematic of light pulse detected by the SSC detector of the flow cytometer when a particle such as a sphere or nanowire passes the laser and disperses light. The collected light pulse has intensity (SSC-Height) and duration (SSC-Width). The two recorded values are integrated to calculate the area (SSC-Area) of the light pulse.

As each particle interacts with the laser beam, the scattered light pulse is collected by the SSC detector and the height, width and area of the pulse is recorded. The gating strategy is built around the SSC-W value taken from each recorded pulse. In this novel gating

strategy, evenly sized gates (corresponding to 10,000 on the SSC-W linear scale) (Figure 3.39, P1-P25) were evenly placed along the SSC-W histogram plot so as to cover the entire histogram range. Any event recorded during acquisition would therefore fall into one of these gates. Data captured on the gated histogram plot was mirrored into the SSC-H vs. SSC-A bi-linear plot for subsequent slope analysis (see section 3.2.12.5). Additionally, the number of events falling in each gate (example in Figure 3.38, inset table, right) was recorded for % distribution analysis in table 3.5.

The data below shows an example of the response from 1 μm polystyrene spheres and the resulting reflection into the SSC-H vs. SSC-A bi-linear plot. The inset table examples the population distribution for each gate. The percentage population was calculated from these values and data was subjected to 2% cut-off threshold. The example above shows >98% of the events fell in P1 and P2, in the case of the microspheres. The results of the gating strategy applied to the nanowire populations can be seen in figure 3.39 and the calculated percentage parent data for nanowires is shown in table 3.5.

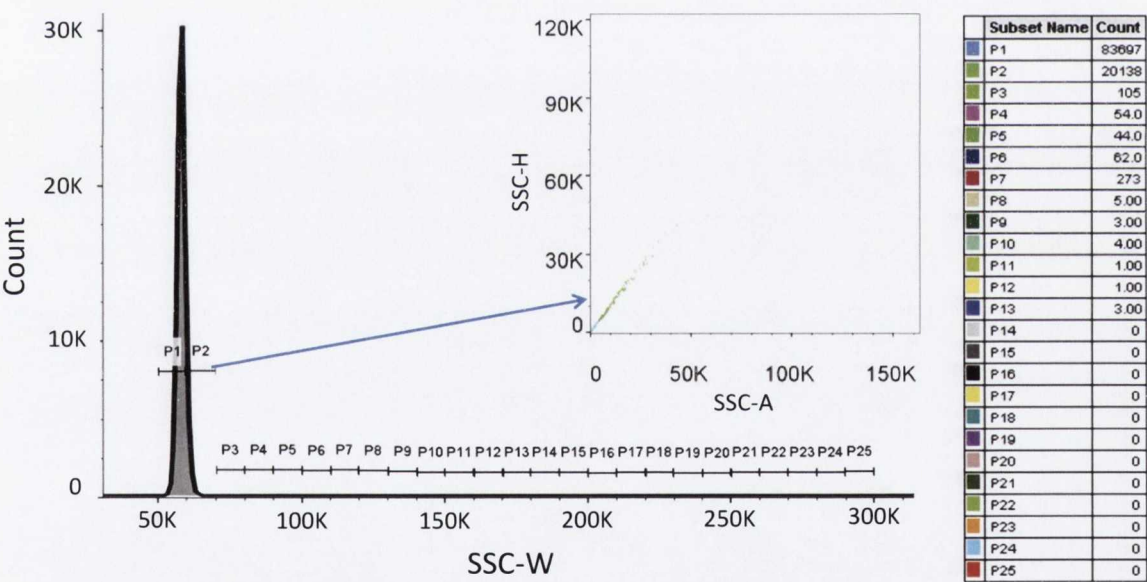


Figure 3.39 Gating strategy for analysis of length-dependent optical response in nanowire populations. Regular gatings (P1-P25) were applied to a SSC-Width (SSC-W) histogram and each gate was then projected into a SSC-Height (SSC-H) vs. SSC-Area (SSC-A) bi-linear plot. The number of events falling into each gate was recorded (right table) for further analysis. This example gating was performed on 1 μm spheres.

3.2.12.4 Analysis of AgNW length associated responses using SSC-W gating strategy.

The custom gating strategy (see section 3.2.12.3) was performed on Ag nanowires batches (2 μ m, 5 μ m, 21 μ m & 26 μ m nanowires) and the data below displays the response of the wires (Figure 3.40 (a)-(d), respectively) in the reflected SSC-H vs. SSC-A bi-linear plot. It was observed that for batches of wires with longer median length, the light scatter response spread into more SSC-W gates, with the result that the event distribution was over a larger area in the SSC-H vs. SSC-A bi-linear plot. The arrows indicate the direction in which the gates P1 to P22 are sequentially mapped onto the bi-linear plot. For shorter nanowires (2 μ m & 5 μ m, (a) & (b), respectively), the events mainly fell into the first four gates (P1-P4) of the histogram (see table 3.5 for details). The longer nanowires (21 μ m & 26 μ m, (c) & (d), respectively) were distributed between many more gates and a sizeable population was noted in P22.

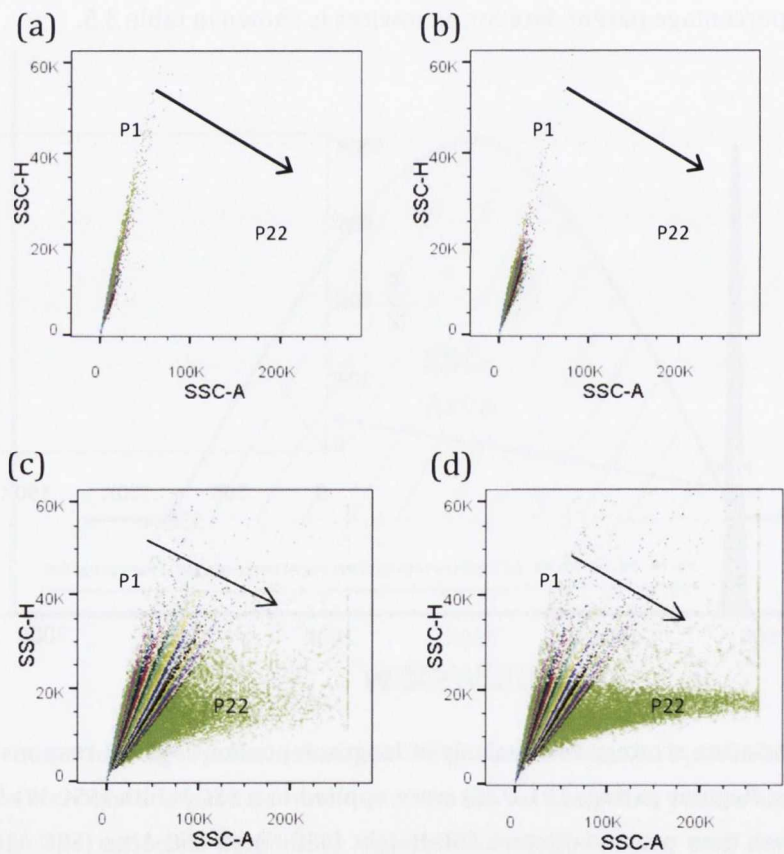


Figure 3.40 Analysis of AgNW length associated responses using SSC-W gating strategy. Regular gatings (P1-P25) were applied to a SSC-Width (SSC-W) histogram for each nanowire sample (2 μ m, 5 μ m, 21 μ m & 26 μ m are graphs A-D, respectively) and each gate was then projected into a SSC-Height (SSC-H) vs. SSC-Area (SSC-A) bi-linear plot. Arrows indicate the order in which gates P1 to P22 populate the SSC-H vs. SSC-A bi-linear plot.

Data presented in figure 3.40 (c) and (d) shows a tendency of longer nanowire populations to appear in later SSC-W gates. The percentage population falling within each gate (P1-P25 as in figure 6) was recorded in table 3.5. and 32% of events in the 26µm wire batch were recorded in P22 (see table 3.5 for details).

Table 3.5 Percentage population occurring in each gate (P1-P22 of P1-P25 shown) for samples A-D. P23-P25 contained no data and so were omitted from the below table.

Nanowire	ID	Gate																					
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
2um	A	22	76	2																			
5um	B	15	60	16	6	2	1																
21um	C	10	16	7	8	5	4	3	3	3	3	3	3	3	3	3	2	2	2	2	1	1	13
26um	D	6	14	8	6	4	4	3	3	2	2	2	2	2	2	2	1	1	1	1	1	1	32

The data in table 3.5 indicates that a proportion of the longer nanowire populations appear in the latter gates in the SSC-W histogram, and shows that the gating strategy may be suitable for separating longer nanowire populations from shorter nanowire populations.

3.2.12.5 Curve fitting calculation for AgNW optical response

Experimental observations noted that, when viewed in SSC-H vs. SSC-A bi-linear plots, spherical populations generally maintained a linear signal response between the two parameters. For anisotropic particles such as wires, and in particular for longer wires, it was observed that the relationship between SSC-H and SSC-A signal magnitude departed from a proportional linear relationship as the wire length increased. In order to quantify this phenomenon, the linear slope data (Figure 3.41) was calculated for sphere populations (a) and wire populations (b)-(e) (slope data shown for spheres and nanowires in left and right tables, respectively).

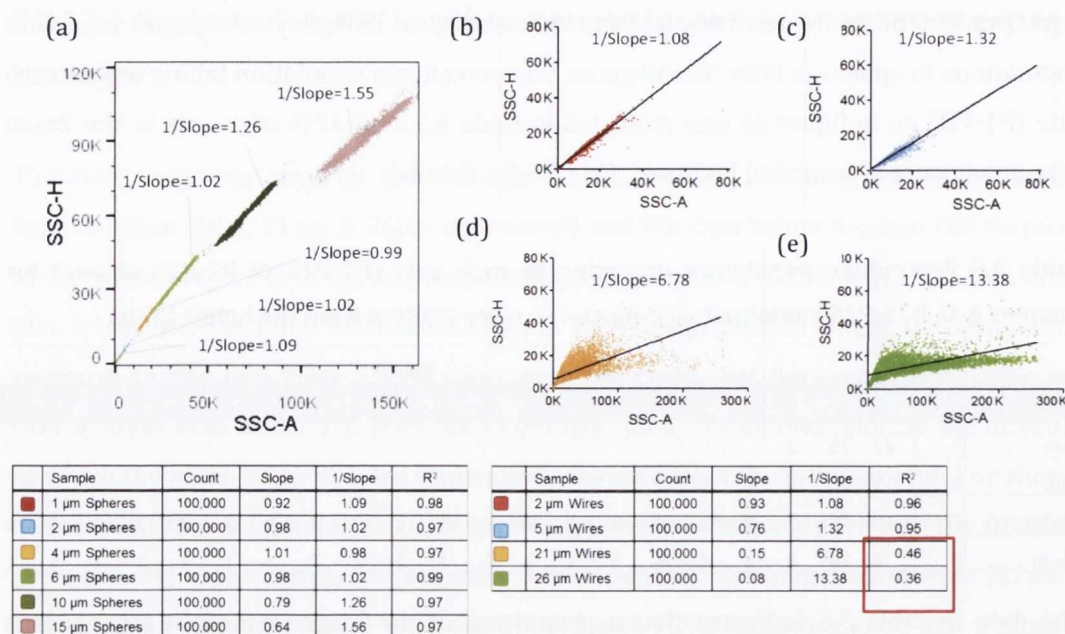


Figure 3.41 Flow cytometric analysis of spheres and wires. Flow cytometric analysis of spheres (A) and wires (B-E) was conducted using the SSC-Height vs. SSC-Area bi-linear plot. 100,000 events were collected for each sample using identical cytometer settings. Data is displayed with inverse slope of linear regression applied to each data set. Slope data for spheres (left table inset) and nanowire (right table inset).

Calculations show that for microspheres, and especially for smaller microspheres, the relationship between SSC-H and SSC-A magnitude for each event was proportionally the same (i.e. the slope of the line for each of the microsphere populations was close to 1). This was also the case for the smaller nanowires, which exhibited a proportionally linear response between the two parameters for the events measured. The larger nanowires however, did not maintain a linear signal response between SSC-H and SSC-A for recorded events. For longer nanowires, the SSC-A signal increased more than the SSC-H signal, indicating that maximal signal intensity (i.e. SSC-H signal) was reached, but the duration of the pulse (i.e. SSC-W) was extended, resulting in the increase in SSC-A signal. The slope calculations support this observation, as the longer nanowire populations have a much smaller slope (or much larger inverse slope) compared to small nanowire and microsphere populations. Indeed the R² value (0.36) associated with the slope data presented in figure 3.41 (e) suggests that use of a non-linear curve fit might be more suitable for long nanowire populations.

The data presented suggests that the gating strategy applied to the interrogation of nanowires could differentiate long nanowire populations from short nanowire populations and may provide a method for improving the interpretation of nanowire-associated optical signals in a flow cytometric setup, with implications for future nanowire assay development.

3.2.13 Comparison of particle detection in custom-built acquisition system vs conventional flow cytometer.

The SSC properties of a range of spheres of different diameters (100, 200, 280, 400, 600 & 3200 nm) were measured in the RDS-CLX system (Figure 3.41 (a), below) and a conventional flow cytometer (Figure 3.42 (b), below). Particle acquisition was triggered on the side scatter height (SSC-H) parameter and 2000 events were captured for each sample. Both sets of data were analysed using RDS-CLX software (Radisens Diagnostics, Ireland). The histogram plots demonstrate that the sensitivity of the RDS-CLX system (a) allows for better separation of particle sizes, especially at small diameters, compared to the conventional flow cytometer (b).

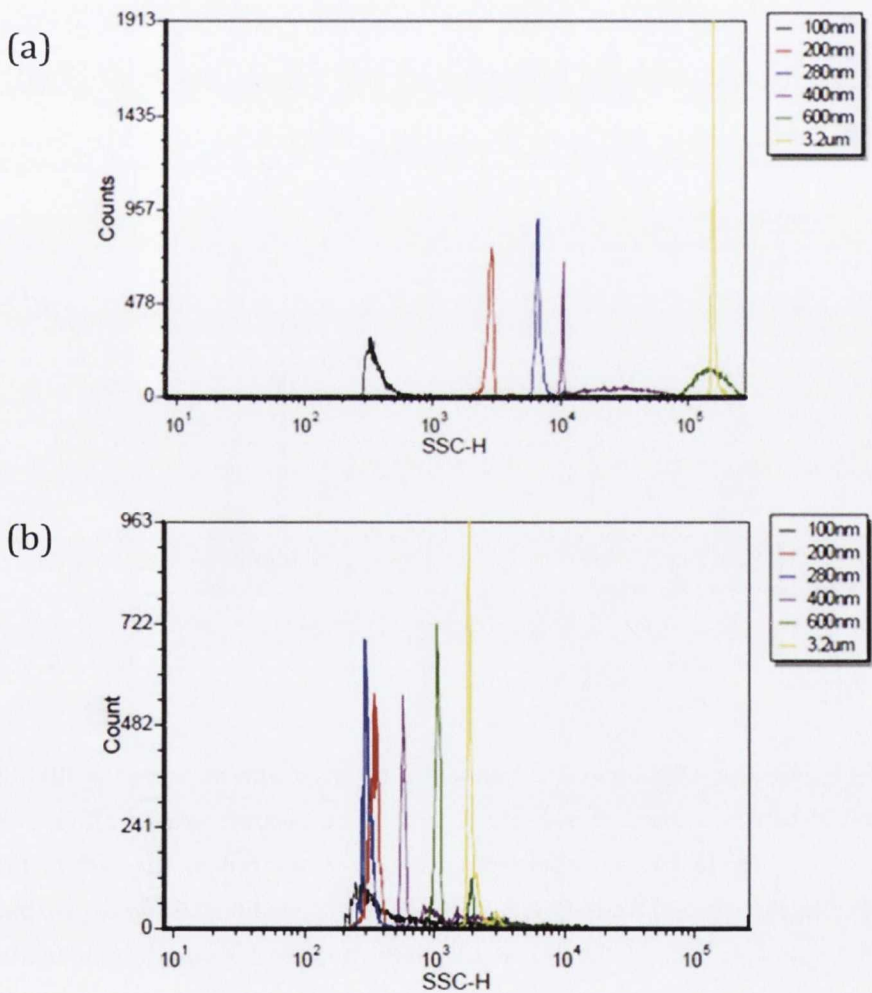


Figure 3.42 Analysis of particle size ladders using custom built and conventional devices. Particle events were recorded using the RDS-CLX system (a) or a conventional flow cytometer (b). Data shows that the RDS-CLX system is much better for distinguishing particles at the smaller end of the sphere size range whilst the conventional system struggles to differentiate between 100nm, 200nm and 280nm particles.

3.2.13.1 Detection of standardised fluorescent microspheres using the RDS-CLX system

The ability of the RDS-CLX system to detect fluorescent events was assessed using commercially available 3.8 μm and 7.3 μm fluorescent microspheres which fluoresced at 500 nm wavelength. Particle acquisition was triggered on the FITC-W parameter and 10,000 events were recorded. The 3.8 μm microspheres were first measured alone (Figure 3.43 (a)) and the fluorescent peak duration (FITC-W) data was plotted on a histogram. The data shows that fluorescent events could be detected by the acquisition system. Furthermore, when the 3.8 μm microspheres were measured in tandem with the 7.3 μm microspheres, the system could detect two separate populations based on the difference in FITC peak duration (Figure 3.43 (b)). These data show that the RDS-CLX system was capable of detecting fluorescent events and the system could be used to discriminate two separate populations of microspheres based on the FITC peak duration (width).

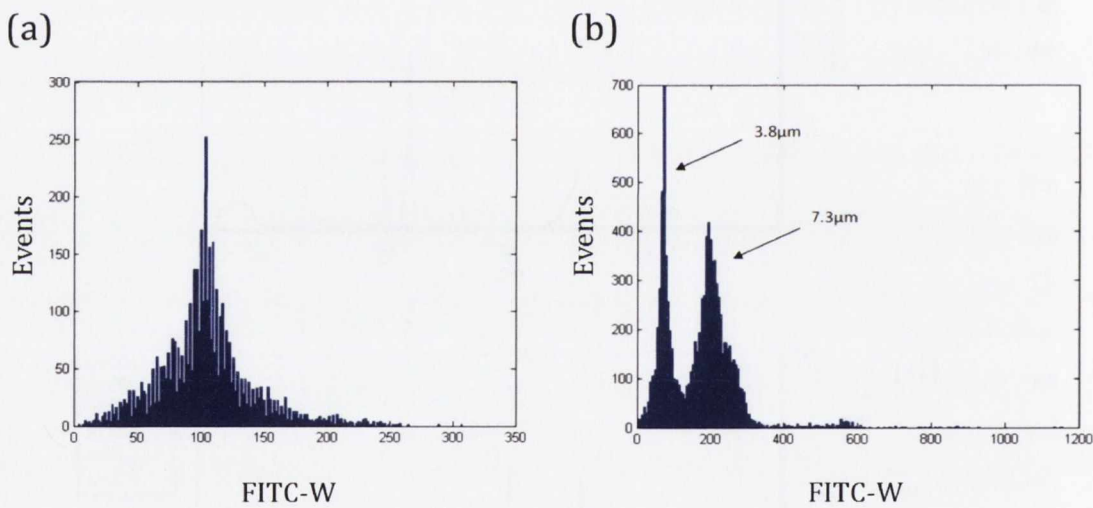


Figure 3.43 Detection of standardised fluorescent microspheres using the RDS-CLX optical fluorescent detection system. The ability of the RDS-CLX system to detect fluorescent events was tested. 3.8 μm fluorescent microspheres were measured by the system (a), and the peak duration distribution in the FITC-W channel was visible. 3.8 μm microspheres were measured in tandem with the 7.3 μm microspheres and the system was able to discriminate between two separate populations based on FITC peak duration, with the larger microparticles exhibiting a longer peak duration response.

3.2.13.2 Detection of fluorescent nanowires using the RDS-CLX system

The RDS-CLX system was successfully used to measure fluorescent antibody-tagged CoNi nanowires. Particle acquisition was triggered on the FITC-W parameter and 10,000 events were recorded. The data shows that fluorescent events could be detected by the acquisition system. The system was also tested with non-fluorescent nanowires and these events could not be read using the FITC channel, indicating that the system was detecting the nanowires based on the fluorescent output of the conjugated fluorescent antibodies. These data show that the RDS-CLX system was capable of detecting fluorescently-tagged nanowires in a flow system using the FITC-W parameter.

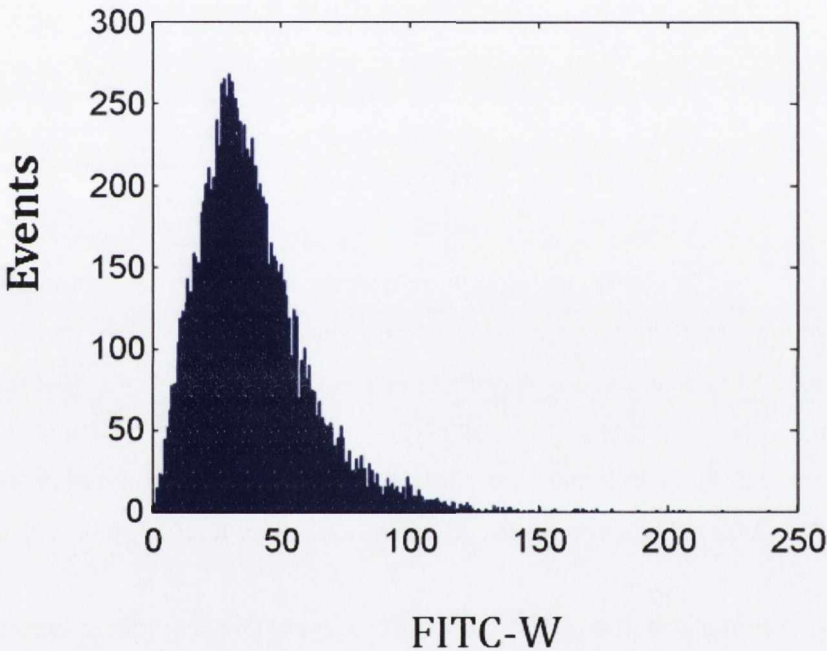


Figure 3.44. Detection of fluorescent nanowires using the RDS-CLX optical fluorescent detection system. The ability of the RDS-CLX system to detect fluorescently tagged nanowires was tested. Fluorescently tagged nanowires were measured by the system and the peak duration distribution was plotted in the FITC-W histogram.

3.3 Discussion

In this results section, the validation of silica nanomaterial-based biological probes is presented, as well as the proof-of-concept demonstration of the ability of the nanowire platform to detect the sErbB2 cancer biomarker in a single and complex analyte sample. Crucial aspects of the development of any new technology are the validation of its internal components and the results suggest that the antibody-nanomaterial conjugates are uniformly functionalised and stable under storage for at least one month. Furthermore, the initial testing of the custom-built microfluidics and fluorescence detector system shows improvements over a conventional flow cytometer setup in terms of size differentiation for particles with nanoscale dimensions. Finally, interrogation of anisotropic Ag nanowires using flow cytometry has yielded some interesting results which may more readily facilitate the future incorporation of nanowires into high through-put flow-based biomarker assays.

3.3.1 Assessment of antibody performance

As part of the validation of the components of the developing nanomaterial-based biological probes, the utility of the selected antibodies was a primary consideration. Of the antibodies (Trastuzumab & AlexaFluor488-anti-CD340 [324402, Biolegend, USA]) selected for the initial proof-of-concept platform, Trastuzumab is a clinically-validated and well-documented ErbB2-specific monoclonal antibody with a defined protein binding site. Anti-CD340-AlexaFluor488 on the other hand is a commercial anti-ErbB2 antibody and is not validated for clinical use. It was necessary therefore to test the anti-CD340 antibody performance against ErbB2 positive cell lines. The cell lines themselves had to be validated for ErbB2 expression and this was done using western blotting and ELISA.

Cell morphology data from bright-field microscopy show that breast cancer (BT-474, SK-BR-3 & MDA-MB-231) and lung cancer (A549, H596 & H520) cell lines were cultivated and achieved characteristic morphologies prior to use in validation experiments. The western blotting assessment of the cell lines for ErbB2 showed a range of ErbB2 expressers with BT-474 and SK-BR-3 cell lines expressing the most ErbB2. Additionally, ELISA assessment of ErbB2 levels not only confirmed BT-474 and SK-BR-3 as high expressers of ErbB2, but the proportional ErbB2 expression in the other cell lines was mirrored between both ELISA and Western blot data. The validation of the cell lines in this manner was necessary before assessment of anti-CD340-AlexaFluor488 performance.

Each of the validated cell lines was immuno-stained with anti-CD340-AlexaFluor488 and staining was measured using confocal microscopy. The results in the breast cancer cell lines showed that immuno-stained BT-474 and SK-BR-3 cell lines exhibited a strong fluorescent signal in the FITC channel whilst no observable fluorescent signal was measurable from the MDA-MB-231 cell line. Immuno-staining of the lung cancer cell lines also showed little or no fluorescent signal in the green channel. These results taken together suggest that the anti-CD340-AlexaFluor488 antibody binds to the cell lines BT-474 and SK-BR-3 which express high ErbB2 levels, but not to cells which express a moderately low or negligible level of ErbB2. Thus the anti-CD340-AlexaFluor488 antibody might not be the most ideal antibody to use for a diagnostic purpose, however, due to the desirable fluorophore quality associated with AlexaFluor488 and the ability of the antibody to bind to ErbB2-positive cells, it was decided that the antibody was suitable for use in an initial proof-of-concept model system, to be used in tandem with Trastuzumab.

3.3.2 Characterisation of bulk nanomaterials

Characterisation of bulk nanomaterials is integral to the development of a new diagnostic assay as the quality of the starting material will ultimately determine the utility of the assay. Therefore stock silica nanomaterials (chosen for their availability, ease of synthesis and functionalisation potential) were characterised by a number of complementary techniques in order to assess the starting material.

3.3.2.1 Nanowire characterisation

Qualitative assessment of silica nanowires by TEM and HIM allowed nanowire structure (see figure 3.12 showing impurity), length distribution and nanowire uniformity (Figure 3.11) to be visualised. Size distribution data obtained from the HIM images showed the silica wire population to have a mean length of 2.79 μm , with a relatively large standard deviation of 1.13 μm (Figure 3.12 (b)). The TEM image gave a rough estimate of 100 nm diameter for the silica wires, but due to the low number of measurements and the visible inherent fluctuation of diameter noticeable in the HIM images, this measurement was not overly relied upon.

In general, the metallic nanowires used in this work had tighter length dimension ranges than the silica nanowires. SEM imaging of CoNi nanowires showed that the nanowire

population was highly uniform in terms of length frequency distribution, with a much smaller relative standard deviation compared to the silica nanowires. The CoNi nanowires had a mean length of 9.65 μm and a standard deviation of 1.35 μm . Additionally, SEM imaging of the Ag nanowire samples showed that Ag nanowires fell within a narrow length distribution range for the smaller size wires (sample (a) and (b) were mean length $2.23 \pm 0.69 \mu\text{m}$ and $4.96 \pm 1.44 \mu\text{m}$, respectively), whereas the longer Ag nanowires had a large size distribution (sample (c) and (d) were mean length $22.69 \pm 13.35 \mu\text{m}$ and $35.38 \pm 23.29 \mu\text{m}$, respectively).

In terms of signal processing of nanowire signatures in a developing diagnostic platform, increasing the uniformity and restricting the length variation of nanowires would certainly go towards improving the overall applicability of nanowires and may reduce associated signal processing challenges (for example, in identifying individual wires in a flow system based on pulse duration). Owing to this consideration, the metallic nanowires of narrow length range would be preferable for the finalised platform, but for the proof-of-concept demonstration, the inherent size and shape variability observed in the silica nanowire populations was a manageable drawback. Added to this, the availability, ease of functionalisation and low cost of synthesis of silica wires made them an attractive starting material with which to work.

3.3.2.2 Nanosphere & microsphere characterisation

Qualitative assessment of polystyrene nanospheres using SEM (Figure 3.15) showed each population to be of uniform shape and within a very narrow size range on the micron scale. Indeed, the calculated size data matched closely the data provided by the manufacturer (Appendix F). SEM sizing of silica spheres showed the mean diameter to be 99.49 nm. The spheres showed good uniformity and the standard deviation was approximately 10% of total diameter. SEM analysis of nanospheres and microspheres conclude that the populations are highly uniform. Silica nanospheres were used primarily as a comparator to silica nanowires in order to compare the difference in analytical approaches needed to assess functionalisation of materials of similar composition but differing geometry (ie. silica spheres vs silica nanowires).

3.3.3 Functionalisation of nanomaterials with antibody

3.3.3.1 Population assessment of nanomaterials: Determining concentration of nanomaterial suspensions

Nanoparticle Tracking Analysis (NTA) was used to measure the hydrodynamic size range and concentration of the silica nanosphere population prior to functionalisation. NTA (Figure 3.18) shows that the stock nanosphere population is of uniform diameter (mode = 106 nm), which correlates favourably with the SEM sizing data. Unfortunately, NTA software has not been developed to track anisotropic nanowires in solution. Therefore, a flow cytometric approach was developed in order to examine nanowires on a population basis. Flow cytometric analysis of silica nanowires (Figure 3.17) showing a wide scatter profile supports the HIM images showing a nanowire population with variable length and uniformity. It was also possible to estimate the concentration of the nanowire population prior to use, a useful assessment which helped to standardise the protocol for nanoparticle functionalisation.

3.3.3.2 Observation of EDC reaction temperature by flow cytometric approach

The developed flow cytometric approach to nanowire analysis proved somewhat useful in tracking the proportion of nanomaterials exhibiting a fluorescent signal, an indirect measurement of antibody conjugation to the nanomaterial surface. By removing a small volume of the reaction mixture at regular intervals and analysing the nanowire population by flow cytometry, it was possible to correlate conjugation efficiency with measured temperature change at each time point. These observations revealed a correlation between reaction temperature and conjugation stability. The data presented in figure 3.20 suggests that the stability of the conjugated nanomaterials is compromised above 32° C. It is possible to speculate that above this temperature, the thermal energy of the system coupled with the sonication energy was sufficient to break the bonds attaching the antibody to the nanomaterial surface, however this speculation was not followed up with any further empirical data. The results obtained from this data supported conjugation reaction temperature as an important factor in effective nanomaterial conjugation and all subsequent nanomaterial functionalisation preparations were carried out below 30° C.

3.3.4 Assessment of nanomaterial functionalisation

Following antibody conjugation to the nanomaterial surface, the validation of antibody-functionalised nanomaterials was carried out. No technique alone was sufficient to make this assessment, so it was necessary to investigate nanomaterial functionalisation by

multiple approaches. Interestingly, the suite of suitable analytical approaches differed, depending on the geometry of the nanoparticle being used (sphere vs wire).

3.3.4.1 Approaches for assessment of antibody conjugation on nanowires

Fluorescent confocal microscopy

Microscopy was successfully used to visualise fluorescent antibodies on the nanowire surface (Figure 3.21). Measurement of the line histogram fluorescence intensity of non-functionalised, fluorescent antibody-functionalised and non-fluorescent antibody-functionalised nanowires showed a marked and obvious difference in the fluorescent signal coming from each wire batch. This data supports the hypothesis that the EDC conjugation reaction resulted in the immobilisation of antibody on the surface of the nanowire. Additionally, the possibility that any observed fluorescent signal was as a result of the antibody alone was ruled out by examination of a non-fluorescently labelled antibody in the same manner. The quantification of mean fluorescence intensity of fluorescent antibodies on nanowires was used later on to compare fluorophore quality between several candidate fluorophores during the development of the nanoprobe platform (discussed separately in section 3.2.11.3). This fluorescence-based approach gives a reliable indication of the presence of antibodies on the nanowire, and gives an assessment of the distribution of the antibodies on the nanomaterial surface.

Atomic Force Microscopy

In addition to optical analysis of the antibody on the nanomaterials by fluorescence-based microscopy, physical confirmation of antibody coverage on the nanomaterial surface was sought through atomic force microscopy (AFM). Topographical assessment of stock and functionalised nanowires by AFM revealed a physical change in surface roughness, and qualitative assessment of antibody coverage could be made (Figure 3.22). Attempts were made to measure the diameter of each wire preparation and indeed, an increase in nanowire diameter post-functionalisation was noted (examples presented in figure 3.22 (b), lower left panel). Due to the labour intensive nature of AFM, it was impractical to measure the requisite number of nanowire diameters (multiple measurements per wire would also be needed to try to account for the inherent diameter variability of the synthesised wires) in order to calculate an average increase in nanowire diameter. However, the AFM results did prove useful in judging the uniformity of antibody coverage

on the nanowire. The antibody coverage was deemed to be generally uniform along the nanowire surfaces.

Helium Ion Microscopy

HIM provides very high resolution (down to ~ 10 nm) imaging and so nanowire functionalisation was examined by this technique (Figure 3.23). Comparison of HIM images of non-functionalised, APTES-coated and functionalised nanowires revealed an increase in surface roughness in the latter two groups. The surface roughness was most pronounced in the antibody-functionalised group (Figure 3.23 (c)). Although this is indicative of antibody being deposited on the nanowire surface, the magnitude of the contribution of APTES to the surface roughness cannot be under-estimated. Therefore, although a promising level of detail was gained from the HIM approach, HIM of the samples as presented are not ideal to conclusively confirm antibody on the surface.

Assessment of antibody conjugation on individual nanowires by a number of complementary approaches suggests that the antibodies were immobilised on the nanomaterial surface. Confocal microscopy probably provided the most convincing data, however no one approach alone should be relied upon to confirm the antibody conjugation. Each of the investigated approaches brought a different strength to the analysis of antibody-nanowire conjugation, with results which together strongly support the conclusion that the antibodies are immobilised on the nanowire surface.

Flow cytometry

The aforementioned approaches give us valuable information on individual members of a particular nanomaterial population, however they are not suitable for assessing the functionalised nanomaterial population as a whole. Nanowires have an optical scatter profile above the noise region and so, not only can confirmation of the presence of fluorescent antibody on the nanowire surface be achieved, additionally, a quantitative assessment of the number of functionalised nanowires as a proportion of the total measured population (non-fluorescent + fluorescent nanowires) can also be determined (Figure 3.24). Therefore the functionalised nanowire population was examined using flow cytometric analysis. This approach compared the nanowire populations pre- and post-functionalisation in comparison to the fluorescent antibody alone. The results show that after conjugation with fluorescent antibody (and several rigorous wash steps to remove

any unbound antibody), the examined population in the conjugated nanowire group shows an increased signal in the FITC channel, suggesting that the nanowires had antibody coupled to them following EDC coupling reaction. In addition, it was possible to quantify the %population which had a fluorescent signal above baseline levels (nanowire population alone), in effect giving a readout of the proportion of functionalised nanowires in a given preparation (43.3% in the given example). This is supported through comparison of the FITC-H Mean for the P1 region of the graph for the purified fluorescent antibody (mean = 3025), non-functionalised (Mean = 564), and fluorescent antibody-functionalised (mean = 2057) nanowires.

A flow cytometric approach was also used to assess the stability of the functionalised nanowire population over a 28-day period. The nanowire preparation was measured on day one of functionalisation and on each subsequent week. The data shows that >99% of the fluorescent signal was located in the quadrant containing the nanowire population, compared to the quadrant occupied by free antibody. This confirms that the antibody did not dissociate from the nanowire surface over a 28-day period. The use of flow cytometry in this way adds to the applications of the approach to nanowire functionalisation analysis and may provide an effective way of batch analysis in large scale functionalised-nanowire production.

3.3.4.2 Approaches for assessment of antibody conjugation on nanospheres

Fluorescent microscopy

Fluorescence-based microscopic analysis (Figure 3.26) was carried out in order to visualise the fluorescent antibody on the surface of the nanosphere. It was possible to detect and record a fluorescent signal from nanospheres and the presence of antibody was confirmed by quantifying the change in mean fluorescence intensity between stock and functionalised nanomaterials. It should be mentioned that in all optical microscopes, including conventional confocal instruments, resolution (defined as the minimum separation between two points that results in a certain level of contrast between them) is restricted by the numerical aperture of optical components and by the wavelength of light, both incident (excitation) and detected (emission) with shorter wavelength radiation allowing better resolution.

Nanoparticle Tracking Analysis

Due to the small size of the nanospheres (100 nm core diameter), the particles were an ideal size for assessment using the Nanosight NS500. NTA was used to measure the hydrodynamic diameter of the nanospheres and proved useful in assessing the uniformity and change in mode particle size of the entire population following functionalisation (Figure 3.27). It was hypothesized that antibody attachment to the surface of the nanosphere would cause an increase in the overall diameter of the nanosphere. The results show a shift in the mode nanosphere diameter from 101 nm to 142 nm (Table 3.2). Assuming the average size of an antibody to be 10 nm, the mode size change suggests that antibodies have caused an increase in nanosphere diameter, thus supporting the notion that the antibodies were present to the nanosphere surface. Additionally, NTA could be used to assess the quality of the nanosphere functionalisation in terms of nanosphere aggregation following functionalisation. The NTA results revealed that the EDC antibody conjugation approach caused some particle aggregation to occur, as denoted by peaks between 280 nm and 500 nm (Figure 3.27 (b)). However, overall the uniformity in the sample population was maintained.

Flow cytometry

The stock 100 nm silica spheres have an optical scatter profile located in the noise region of the flow cytometer, so they could only be discriminated from noise through presence of a fluorescent signal (i.e. when conjugated with fluorescent antibody). In this case, only the concentration of functionalised nanospheres can be estimated. It is useful however, to observe that in the antibody-functionalised nanosphere group, events could be viewed in the fluorescent channel. Since we assume all free antibody was washed away post-functionalisation, the data could support the conclusion that these fluorescent events represent the functionalised nanospheres. Realistically, the fluorescence-based assessment of functionalised particles using the provided flow cytometric setup is more reliable for particles of larger size (>200 nm diameter) or sub-200 nm diameter particles with a higher optical density than silica, where the scatter profile of the non-functionalised stock particles could be easily distinguished from background noise. For this reason, flow cytometry was not ideal for assessing the coupling efficiency of antibody to 100nm silica nanospheres, and other approaches were investigated.

Spectroscopy

Spectroscopic analysis of nanosphere populations was investigated as a means of confirming fluorescent antibody immobilisation on the nanosphere surface. This approach is based on the known peak absorbance wavelength of the fluorophore, which

in the case of AlexaFluor488 is known to be 495 nm. Non-functionalised nanospheres, functionalised nanospheres and purified antibody were spectroscopically compared. Each sample was excited with incrementally increasing light wavelengths (300 nm – 800 nm) in 10 nm increments and the corresponding absorbance was measured (Figure 3.29). The results show a clear absorbance peak in both the purified fluorescent antibody (green line) and the functionalised nanosphere sample (blue line), but not in the stock nanosphere sample (brown line). Both of the absorbance peaks aligned at the same wavelength even though the background absorbance measured in the functionalised nanosphere sample was much higher. Although this result does not give detailed data on the population subtypes (compared to NTA and flow cytometry), it does further support the conclusion that the antibody is present on the surface of the nanospheres and may serve as a quick method of assessing the success of a nanomaterial functionalisation preparation.

Gel electrophoresis

Gel electrophoresis was investigated as a means of assessing the entire functionalised nanosphere population. The approach was not suitable for assessing nanowires as their geometry would cause them to become trapped in the gel. Stock and functionalised nanospheres were electrophoresed in gel and the progress of each population was recorded (Figure 3.30). The results show that non-functionalised nanospheres migrated toward the cathode and the functionalised nanospheres remained at the loading point. It is suggested that either a change in surface charge or change in nanosphere diameter could cause the nanospheres to remain at the loading point. Considering the observation that the functionalised nanospheres appear to align along the left-hand side of the loading well coupled with the observed change in mode diameter recorded by NTA previously discussed (Figure 3.27), it is concluded that the retardation of the nanospheres in the well is more likely due to a change in nanosphere diameter caused by antibody adherence to the surface.

3.3.4.3 Assessment of antibody conjugation on nanomaterials

The initial development of a proof-of-concept nanomaterial platform for the detection of biomolecules required a feasible method of conjugating a desired antibody to a nanomaterial surface, followed by the confirmation of validation of that conjugation as one that produced stably conjugated nanoprobe. The EDC-driven antibody coupling reaction was used to attach antibodies to silanised silica nanomaterials of differing geometries, namely nanowires and nanospheres. A number of different approaches were investigated as to whether they were useful for (a) confirming antibodies on the surface

of the nanomaterial, (b) estimating the functionalisation efficiency of the reaction and (c) assessing the stability of the bio-conjugates over time.

A number of approaches, namely fluorescence-based microscopy, atomic force microscopy and helium ion microscopy, were used to interrogate individual nanowires post-functionalisation with antibody, and the results strongly support the conclusion that fluorescent antibodies were present to the nanowire surface following functionalisation. In addition, the developed flow cytometric approaches to assessing nanowire populations for antibody functionalisation efficacy (judged as %population of nanowires carrying a fluorescent signal) and stability (judged as the %population of fluorescent events migrating from the nanowire quadrant to the free antibody quadrant [$<0.2\%$]), gave a more accurate assessment of the success of the EDC coupling reaction.

Due to the differing geometry of nanowires and nanospheres, it was not really feasible to assess nanosphere functionalisation success using the same parameters as those used for nanowires. Although fluorescent nanospheres could be measured using fluorescence-based microscopy, due to their small size, the 100 nm silica particles were more usefully assessed by less high-resolution approaches including nanoparticle tracking analysis, spectroscopy and gel electrophoresis. Flow cytometry could be used to a limited extent for nanosphere assessment, however it was not an ideal approach to use due to the fact that non-functionalised silica nanospheres could not be accurately differentiated from the inherent noise of the available flow cytometer, based on scatter characteristics alone.

All results considered, the EDC-driven coupling of antibodies to silica nanomaterial surfaces resulted in stable bio-conjugates. The functionalised nanomaterials were sufficiently well validated to allow progress to the next step in nanoprobe platform development.

3.3.5 Detection of sErbB2 using developed nanomaterial-based platforms

Following validation of the EDC-functionalisation of nanomaterials, the functionality of the nanowire platform was tested. In this case, nanowires were functionalised with a non-fluorescent capture antibody (Trastuzumab) against ErbB2. The functionalised wires were incubated in either sErbB2 or BSA solution, washed, and incubated with fluorescent anti-ErbB2 detection antibody (anti-CD340-AlexaFluor488). Each group was imaged using confocal microscopy and examined using flow cytometry. Confocal microscopy (Figure 3.31) clearly shows that in the presence of sErbB2, fluorescent detection antibody is immobilised on the nanowire. In the presence of a non-specific target, BSA, the fluorescent detection antibody is not immobilised on the nanowire surface. Flow

cytometric data indicates capture of sErbB2 on the nanowire, judging by the presence of the strong fluorescent signal in the FITC channel. Both confocal microscopy and flow cytometry data support the conclusion that this nanowire-based system is able to preferentially capture sErbB2 compared to BSA. Taking into consideration the earlier findings that the affinity of the anti-CD340-AlexaFluor488 for ErbB2 may not be exceptionally strong, it was necessary to use antibody pairs which had high affinity for ErbB2 and shared compatible binding epitopes on the target protein. Comparative work conducted by a partner laboratory concluded that the mouse anti-ErbB2 monoclonal antibody (mAb) (BMS120, eBioscience Ltd, UK) and the complementary goat anti-ErbB2 polyclonal antibody (pAb) (AF1129, R&D Systems, Inc, USA) detection antibody provided the best antibody pair match for ErbB2 detection. With this information, nanowires were functionalised with a FITC-tagged anti-ErbB2 mAb (BMS120FI) as the capture antibody. The anti-ErbB2 pAb detection antibody did not have a fluorescent tag attached, and so a fluorescent secondary antibody (donkey anti-goat pAb - Phycoerythrin) was used to indicate analyte detection on the nanowire.

3.3.6 Detection of ErbB2 concentration range in single analyte system

The nanowire system (using anti-ErbB2 (BMS120) capture Ab + anti-ErbB2 (AF1129) detection Ab) was tested to see if detection of a concentration range of sErbB2 in a simple system was possible. In addition, the specificity of the nanowire-based platform was also tested by functionalising a control nanowire batch with non-specific IgG as the immobilised capture antibody. This control would help to determine whether the nanowire itself was contributing toward non-specific binding events. The nanowires were incubated in various concentrations of sErbB2 (1000 ng/ml, 250 ng/ml, 62.5 ng/ml & 0 ng/ml) followed by incubation with the detection antibody and fluorescent secondary antibody. The nanowires were washed and analysed with flow cytometry and the PE-H Median values (Figure 3.32) were recorded from the nanowire sample. The data shows that the developed nanowire system could successfully detect a concentration range of sErbB2 and that the same platform functionalised with a non-specific antibody did not give a false positive result and comparable concentration ranges. The data supports the suggestion that the anti-ErbB2 nanowire platform could detect an sErbB2 concentration gradient over a relatively large response range (1000 ng/ml – 0 ng/ml). The physiologically relevant concentration range is noted as above 15 ng/ml and can extend towards 200 ng/ml.

3.3.7 Detection of sErbB2 in spiked plasma

Following the detection of sErbB2 in a single analyte system, the performance of the developing nanoprobe systems were tested for their ability to detect concentrations of spiked sErbB2 (100 ng/ml to 0 ng/ml) in blood plasma. Both nanosphere and nanowire systems were tested. As before, FITC-conjugated anti-ErbB2 antibody (BMS120) was used as a capture antibody in order to help to distinguish nanoparticles from background noise during flow cytometry. Detection antibody (AF1129) was visualised using a PE-labelled secondary antibody. The samples were treated as in Table 3.3, and included the use of universal casein to reduce the possibility of non-specific binding events. Functionalised nanoprobe systems were recorded using the FITC threshold in the flow cytometer. Positive signal was registered when a PE signal above background levels was recorded in coincidence with a FITC-labelled nanoprobe. The mean fluorescence intensity from each sample was expressed as [nanoprobe with PE signal] divided by [nanoprobe without PE signal], within each sample. The results (Figure 3.33) demonstrate that both nanoprobe systems showed a response to spiked sErbB2 over a concentration gradient. The nanosphere system (Figure 3.33 (a)) could detect a concentration gradient between 50 ng/ml and 0 ng/ml but could not differentiate between 100 ng/ml and 50 ng/ml. The nanowire system (Figure 3.33 (b)) could detect a concentration gradient between 100 ng/ml and 0 ng/ml, based on mean values, however, the within-treatment group readings were less reliable (notable by the large standard deviation). It was noted that although the overall signal was lower from the nanowire platform, the linearity of response in that system, compared to the non-linear response of the nanosphere platform (especially at the low sErbB2 concentration) was a desirable characteristic of the nanowire platform.

Although nanomaterial-based systems could be used to detect spiked sErbB2 in blood plasma or native blood plasma, the accuracy and reproducibility of readings were deemed suboptimal. The suboptimal performance of the nanoprobe systems could be due to a number of factors including (but not limited to) suboptimal fluorophore performance, user error in assay handling, inherent variation in clinical samples, optical limits of the flow cytometer in determining significant events or a suboptimal data analysis approach. Of these, addressing suboptimal fluorophore performance carried most potential to improve the sensitivity of the assay system. It was noted during experimentation that the FITC fluorophore was not as fluorescently intense as other fluorophores in the same spectrum. There was a possibility that FITC was not intense enough to allow the flow cytometer to differentiate all nanomaterial events from background signals, with the

result that otherwise significant events were being unrecorded. A comparison of fluorophores was carried out to address this issue.

3.3.8 Comparison of fluorophores

Following development and testing of the nanoprobe systems, suboptimal fluorophore performance was identified as the phenomenon most likely to affect the sensitivity of the assay. Therefore quantitative comparison of several alternative fluorophores was undertaken to establish the fact and select a possible suitable replacement (Figure 3.34 and 3.35). Confocal microscopic and flow cytometric analysis of nanowires functionalised with FITC-, CF488- and AF488 (AlexaFluor488) -tagged antibodies revealed FITC to be quite unsuitable for use in a high-fidelity fluorescence based detection system.

Firstly, the three candidate fluorescent antibodies were functionalised on a glass surface (readily available silica substrate), followed by microscopic analysis (Figure 3.34). The results of fluorescence-based analysis were expressed as mean fluorescence intensity (MFI) and the data reveals that FITC-antibody-functionalised glass registered an MFI at least two times less than either CF488- or AF488- functionalised glass. The CF488 and AF488 fluorophores were quite similar in measured fluorescent intensity.

Each fluorophore was then conjugated to silica nanowires and compared. Fluorescent microscopic analysis (Figure 3.35 (a)) showed FITC-nanowires to have an MFI (MFI = 30) much lower than that of either CF488- (MFI = 140) or AF488-nanowires (MFI = 150). Furthermore, flow cytometric analysis revealed that the signal intensity from FITC-nanowires was insufficient to clear the cytometer fluorescence detector threshold (Figure 3.35 (b)) and so it was likely that a significant proportion of nanowires were not being recorded. Conversely, the fluorescence signal from both CF488- and AF488-nanowires was sufficient to clear the threshold. Accordingly, the fluorescence intensity for FITC-, CF488- and AF488- nanowires measured by geometric mean was 448, 3810 and 4477 respectively. The conclusion was made that FITC is an unsuitable fluorophore for use in the developing nanoprobe system and CF488 was selected as the alternative fluorophore for use in the developing nanowire platform.

3.3.9 Analysis of nanowire signatures using flow cytometry

3.3.9.1 Comparison of scatter properties of AgNWs with spherical particles using flow cytometry

AgNWs and spherical particles of various sizes (size distributions for each nanowire and microsphere group were determined from the SEM measurements presented in section D) were analysed in the flow cytometer and the median values of Forward Scatter (FSC-A, FSC-H & FSC-W) parameters as well as Side Scatter Area (SSC-A, SSC-H & SSC-W) parameters were calculated for each population. The results showed that for five of the six parameters (FSC-A, FSC-H, FSC-W, SSC-A & SSC-H), microspheres generated much greater scatter signals than nanowires. This was widely expected, as the microspheres occupy a much larger volume in the flow stream and thus are more likely to disperse incident light. For example, volume calculations for a sphere of diameter 10 μm is around 524 μm^3 whereas a nanowire of similar length and an arbitrary diameter of 0.1 μm is about 0.31 μm^3 . Thus, even though the two shapes share similar dimensions in one plane, the result of their differing geometries means that one (nanowire) is almost invisible in a flow cytometric system. Interestingly, when both sets of particles were viewed in the SSC-W parameter, the results were different. Nanowires of longer median lengths contributed a larger SSC-W signal compared to similarly sized microspheres. This observation led to the idea that SSC-W may be the optimal parameter for viewing nanowire populations and that it may be possible to identify nanowires of varying lengths based on their scatter characteristics.

3.3.9.2 Comparison of SSC-W response of AgNWs and spherical particles using flow cytometry

SSC-W was the only parameter in which the length of the AgNWs correlated favourably with signal output, with wires and spheres of comparable lengths having similarly signal outputs. The flow cytometric data was further visualised using FlowJo software, and the results (Figure X) illustrate the distribution of the events along the SSC-W histogram. The results show that although the smaller nanowires (2 μm & 5 μm length) show SSC-W responses in the same range as the microsphere set, the longer wires (21 μm & 26 μm length) have a much larger SSC-W response. As pulse width readings can be related to the amount of time a particle spends in the interrogating light path, it would seem plausible that longer wires would show a larger SSC-W value. Interestingly, the SSC-W histogram of the nanowires show that the proportion of wires registering a large SSC-W signal

correlates well with nanowire length. Further gating strategies needed to be developed in order to further investigate this phenomenon.

3.3.9.3 Analysis of length-associated response in nanowires using SSC-W gating strategy

Using the custom gating strategy outlined in the methods section, analysis of each of the nanowire and microsphere sets was carried out. For the microsphere sets, the majority of the events occurred within the early gates (P1-4). In addition, when the microsphere populations were reflected into the SSC-H vs. SSC-A bi-linear plot, the result was the appearance of a generally linear population (see diagram D for composite image). This linear response indicated that the increase in SSC-H intensity was matched with a proportional increase in SSC-W (and therefore SSC-A). This indeed might be expected of a spherical particle with isotropic geometry.

Analysis of the AgNWs using the same gating strategy yielded quite different results. For the smaller nanowires, the majority fell within the first three gates (P1-3). However, distribution data (Table 3.5) shows that more of the larger wires were found in later gates (P22). Interestingly, when the nanowire populations were reflected into the SSC-H vs. SSC-A bi-linear plot, this resulted in the migration of the scatter characteristics away from a linear distribution and more toward a dispersed population spread (Figure TY). This is indicative of events which carry a larger SSC-W (and therefore larger SSC-A) but, unlike the microspheres, this increase in SSC-W (SSC-A) is not matched by an increase in SSC-H. In other words, the duration of the pulse increases, without a proportional increase in the intensity of the pulse.

This finding was visualised further by fitting a best-fit line to each of the data sets and recording the slope of the line. For spherical isotropic particles, the slope was generally close to 1 (Figure U), meaning that there is a proportional relationship between SSC-H and SSC-W. For anisotropic nanowires, an increase in median wire length correlated with a decrease in slope, with the longest nanowire population exhibiting a slope of 0.08. Indeed, observation of the low R^2 values (an indicator of confidence in the line fit) of the 21 μm and 26 μm nanowire groups suggest that a non-linear line-fit would be more representative of the data.

From these results it could be postulate that; (a) AgNWs create enough side scatter interference to be effectively measured on the available flow cytometric setup using the

SSC-W parameter (b) The magnitude of the SSC-W signal in AgNWs is greater than that of similarly sized polystyrene spheres (c) Increasing AgNWs length correlates with a more non-linear population distribution in the SSC-H vs. SSC-A bi-linear plot & (d) AgNWs above a certain length may become axially restricted by the laminar flow and so align in the direction of the sheath flow.

This type of nanowire analysis may more easily facilitate the integration of nanowires into high throughput diagnostic platforms in future flow cytometric systems.

3.3.10 Comparison of particle detection in custom-built acquisition system vs conventional flow cytometer

In an effort to build systems which can capitalise on the benefits of incorporating nanomaterials into their setup, a custom-built detection system (a new system, from now on referred to as the RDS-CLX) for nanoparticles was developed by project partners (Radisens Diagnostics and Cellix), specifically for the NAMDIATREAM project. One of the system aims was to improve the ability to distinguish particles on the nanoscale better than the current flow based systems, such as cytometers (which are tuned to track micron-sized particles).

A range of spheres of different diameters (100nm, 200nm, 280nm, 400nm, 600nm & 3.2µm) were analysed in the RDS-CLX acquisition system and a conventional flow cytometer. The results show that the RDS-CLX system is much better at distinguishing sub-micron particles, compared to the conventional flow cytometric setup. The RDS-CLX system could readily distinguish 100 nm and 200 nm calibration spheres, whereas the flow cytometer struggled to differentiate 100 nm, 200 nm and 280 nm particles. This improvement in particle detection could be integrated into future nanomaterial based systems for the detection of submicron particles. Additionally, the RDS-CLX system could successfully detect fluorescently-tagged nanowires, and the recorded FITC-W signal was sufficiently strong to separate the nanowire signals from the noise region on the histogram plot. These measurements show that the developed system can handle both micro-sized particles and nanowires and serve as a basis for continued development of the RDS-CLX system for use in conjunction with the nanowire platform.

3.3.11 Concluding key-points

1. Achieved characterisation, functionalisation and validation of nanomaterial-antibody conjugates.

- a. Confirmed the presence of antibody on the nanomaterial surface.
- b. Assessed antibody distributed across the nanomaterial.
- c. Estimate the proportion of the nanomaterial population which was sufficiently functionalised.
- d. Track the stability of the nanowire-antibody conjugates following functionalisation.

2. Demonstration of specific analyte detection in single analyte samples.

- a. Detected sErbB2 using the nanowire assay

3. Development of novel approaches towards optimal nanowire visualisation in a flow cytometric system.

- a. Identified SSC-W as the most appropriate parameter for viewing nanowire length-associated responses.
- b. Developed a novel gating strategy to analyse nanowire signatures in flow cytometry

4. Demonstration of a custom-made acquisition system for nanomaterial assays.

- a. Improved sensitivity in detection of nanomaterials over conventional flow cytometers
- b. Achieved detection of nanospheres and functionalised nanowires using the setup

3.4 References

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Chapter 4

Results: Part II

Biomarker detection in complex biological
fluids using the nanowire assay

4.1 Introduction

4.1.1 Biomarkers as indicators of disease

Biomarkers can be thought of as objective indications of medical state observed from outside the patient (Strimbu & Travel 2010). Simple examples of clinically observable biomarkers include body temperature and pupillary reflex (Capo-Aponte et al. 2013), and assessment of these fundamental parameters can be used to inform medical decisions and track response to intervention. It is evident even at this most basic level, that mono-parametric evaluation is often inadequate for accurate deduction of health status (for example, body temperature could be elevated by exercise, sickness-induced fever, hormonal changes, environmental conditions or a combination of each). At a molecular level, the term biomarker has been applied to all classes of molecules (including DNA, RNA, proteins, carbohydrates) which are detectable and quantifiable in tissue (through swab or biopsy) or body fluids, see examples in figure 4.1(a).

Whilst examining biomarkers in tissue often requires biopsy (not ideal in instances where access to tissue is restricted, such as in lung tissue), body fluids provide easy access to a rich source of biomolecules, quantification of which can potentially serve as a reliable barometer of patient internal health. Cancer biomarkers can be thought of as predictive (identifying subpopulations of patients who are most likely to respond to a given therapy), diagnostic (a detectable marker in healthy individuals, the quantity of which is altered in the presence of cancer) or prognostic (provides information on the likely course of the cancer disease in an untreated individual) (Brunner 2009) and techniques such as chromatography, mass spectrometry and electrophoresis have contributed to the discovery of a vast and growing database of biomarkers (Leigh Anderson et al. 2004, Li et al. 2009) as illustrated in Figure 4.1 (a).

Biomarker assessment holds a number of advantages for diagnosis and treatment. Firstly, abnormal biomarker levels may precede an observable clinical manifestation, allowing for screening and early intervention in the disease progression. Indeed, recent evidence has supported the need to prioritise the development of early cancer detection methods as the key to reducing cancer related morbidity in what are termed sporadic cancers (Tomasetti & Vogelstein 2015). Secondly, biomarker quantification allows objective comparison over time and facilitates assessment of treatment response. Additionally, a number of biomarkers are detectable in more than one fluid type (Figure 4.1 (b) and figure 4.2) and this may allow certain flexibility in sample collection and processing approaches as well as the ability to assay several analytes simultaneously. For example,

from the data presented in figure 4.1 (b), it is estimated that two fluids share nearly 3000 different proteins and there are close to 2400 shared proteins between three biological fluids. It would be interesting to assess whether each biological fluid type has a similar protein concentration, or whether there is a consistent scaling factor that applies when measuring between different fluids. It is clear from the graphs in figure 4.1 that blood, saliva and urine carry the greatest potential for biomarker assessment, but those fluids with fewer distinct protein may by their very nature, be less complex and easier to handle in an assay setting.

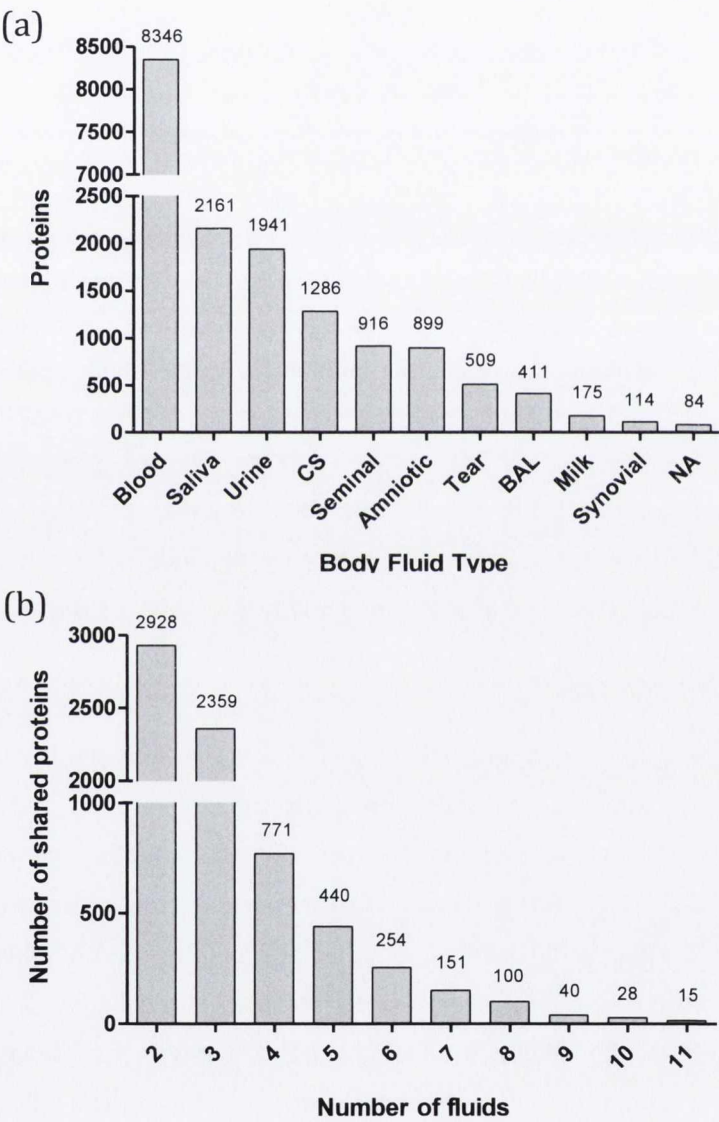


Figure 4.1 Biomarkers in human body fluids. (a) Number of detectable unique proteins per body fluid type using mass spectrometry approaches. This figure shows the number of detectable proteins per fluid type and indicates blood as the richest source of potential biomarkers (8346), ranging two orders of magnitude more identified proteins than nipple aspirate fluid (84). (b) Number of shared proteins in a number of body fluids. This figure shows the potential to measure identical biomarkers in a number of different fluids, and

underlines a consideration that may be taken when choosing a biomarker for multiplexed clinical diagnostic purposes. Figures adapted from (Li et al. 2009) & www.biosino.org/bodyfluid/database.jsp. CS: Cerebrospinal Fluid, BAL: Bronchoalveolar lavage, NA: Nipple Aspirate Fluid.

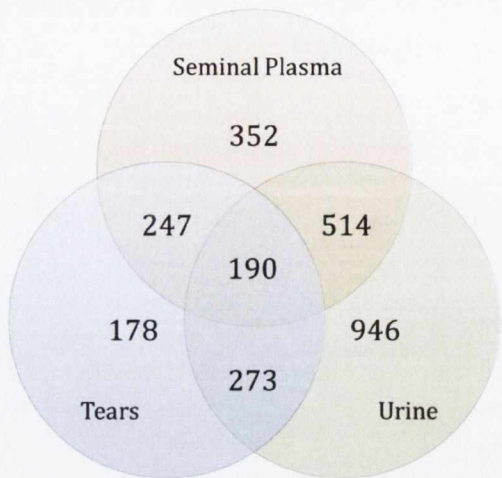


Figure 4.2 Number of similar proteins detectable in different body fluid types. This figure demonstrates the number of similar proteins found between three biological fluid types, namely seminal plasma, tears and urine. The figures illustrate that some biomarkers may be amenable to assay from more than one body fluid, whilst others are restricted in their distribution to one fluid type. Figure adapted from (Schmidt & Aebersold 2006).

It should be noted at this point that the number of proteins identified and exemplified for each body fluid in figures 4.1 and 4.2 is heavily dependent on a number of factors including sample collection and preparation conditions as well as the analytical approach applied towards identifying proteins of interest.

In a clinical setting measured biomarker concentration in body fluids can be affected by many inherent factors such as hydration state, tumour location and vascularisation, protein half-life as well as sampling factors such as sampling method and sample processing. Of these, sampling method and processing are the factors which are directly controllable by the diagnostic team. Such is the nature of the effect of inherent factors that biomarker quantification is most useful when interpreted in the context of an individual's baseline readings. The development of reliable, cost effective cancer biomarker assays for complex biological fluids is often not a straightforward process. The following section will illustrate the challenges faced in accurately identifying proteins in body fluids, especially in blood plasma, and generating a reliable database of proteins for subsequent biomarker selection. Additionally, the necessity of biomarker and assay validation and the place of multiplexing in future cancer biomarker assessment will be mentioned.

4.1.2 Cancer biomarker assessment in blood

The vascular system services all regions of human anatomy and allows for the effective delivery of nutrients, blood clotting factors and immune surveillance as well as the removal of metabolic waste products to and from local tissues. It is the ability of biomolecules to pass from cells into the blood plasma via the interstitial fluid (Figure 4.3 (a)) which allows us to simultaneously survey the health status of the an entire organism by accessing a relatively small volume of blood plasma (~7.5% total body fluid volume. Figure 4.3 (b)). Blood plasma collected from a patient can be readily separated into fluid and cellular components (Figure 4.3 (c)) through centrifugation and it is the plasma component that can then be further processed for soluble biomarker discovery and quantification.

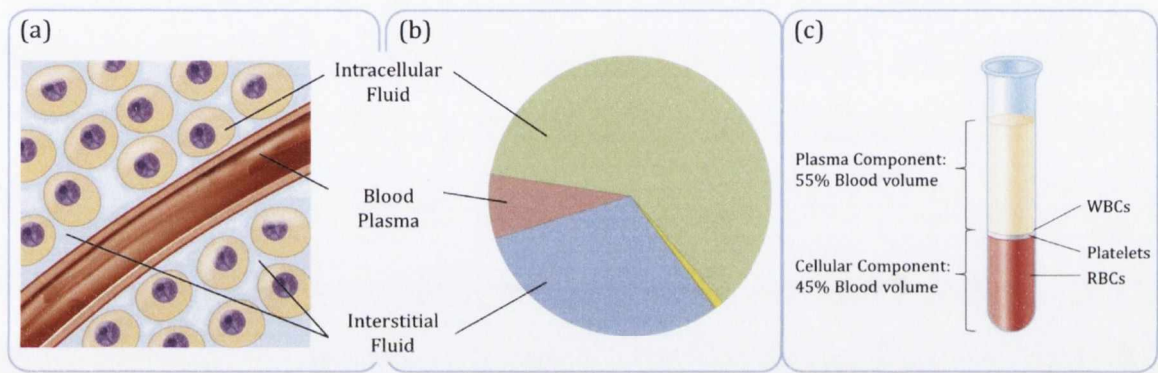


Figure 4.3 Body fluid compartments. (a) Graphic of the main fluid compartments of the body, namely intracellular fluid, blood plasma and interstitial fluid. (b) Pie chart representation of the proportion of total body fluid volume for intracellular fluid (green segment: ~62.5%), blood plasma (red segment: ~7.5%) and interstitial fluid (~30%). Other body fluids such as cerebro-spinal fluid (yellow segment) make up the remaining total fluid volume. WBCs: White Blood Cells. RBCs: Red Blood Cells. Figure 4.3 (a) adapted from cnx.org.

As blood plasma appears to contain the richest source of identifiable proteins (Figure 4.1 (a), above) for biomarker assessment, coupled with the penetration of vascularisation throughout the body (and the enhanced tissue vascularisation induced by tumours (Watnick 2012), it is not surprising that blood is the focus of most of the soluble biomarker studies over the last couple of decades. Interestingly, whilst the complexity and wide dynamic range of proteins in blood plasma provides a wide choice of possible biomarkers, this also poses substantial technical challenges in terms of accurate biomarker discovery and analysis (Dayon & Kussmann 2013).

Serum is the liquid fraction of whole blood that is collected after the blood is allowed to clot. Plasma is produced when whole blood is collected in tubes that are treated with an anticoagulant. The blood does not clot in the plasma tube. The cells are removed by centrifugation to leave the supernatant, designated plasma. In the case of serum, removal of the clot may also remove a subset of proteins which associate with the clot and clotting factors. This may result in a depletion of the biomarker in the measured serum, thus giving an inaccurate result in terms of assessing the actual physiological levels of the biomarker. Therefore, if novel biomarker assessment is being carried out, it should initially be done in both plasma and serum to see whether one liquid yields a higher concentration of the target biomarker.

4.1.3 Developing future cancer biomarker assays for blood fractions

4.1.3.1 Identifying proteins of interest in blood

Even prior to the challenge of protein assay for clinical diagnostics, the initial accurate identification of proteins in blood with high confidence is not a simple task. For example, over the last decade, significant resources have been employed by the Human Proteome Organisation (HUPO) through the Plasma Proteome Project (PPP) in order to detail an extensive, quantitative catalogue of plasma proteins, for subsequent use as a guide for blood-based diagnostic efforts (Farrah et al. 2011, Schenk et al. 2008). Early work by the consortium showed that choice of sample preparation (such as the choice of using EDTA-, citrate-, or heparin-stabilised plasma) and analysis techniques had a large impact on the identified proteome (Rai et al. 2005). Since then, accumulated mass spectrometric analyses (Figure 4.4) of blood samples (containing some 1.9 million spectra) carried out by multiple laboratories have yielded a list of 1929 highly non-redundant protein sequences named the PeptideAtlas (Farrah et al. 2011). Modern peptide identification processes are dependent not only on the method of sample preparation and processing technique, but increasingly on the statistical modelling software used (e.g. in (Keller et al. 2002)) to decipher the complex datasets generated.

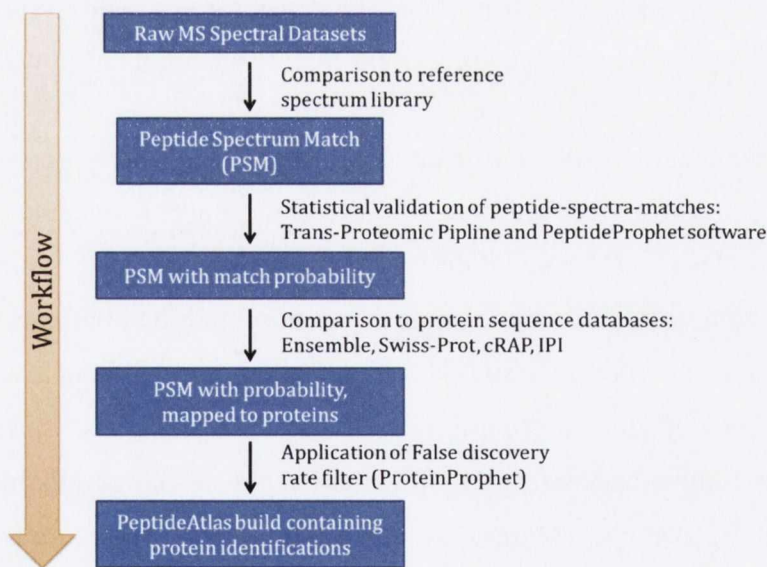


Figure 4.4 Workflow of PPP analysis method used to generate highly non-redundant protein sequences for PeptideAtlas. Raw mass spectrometric (MS) spectra generated from plasma and serum samples were searched against a spectral library or sequence database. The resulting peptide spectrum matches (PSMs) were then statistically validated and high probability hits were matched to existing protein sequence databases, generating a list of mapped proteins. All mapped PSMs passing the false discovery rate filter created a lists of high-confidence protein identifications, known as a PeptideAtlas build. IPI: International

Protein Index. cRAP: Common Repository of Adventitious Proteins. Figure adapted from (Farrah et al. 2011).

4.1.3.2 Validation of novel cancer biomarker assays

Following the selection, validation and approval of cancer biomarkers (usually involving extensive validation studies comprising longitudinal assessment of biomarker concentrations and correlation with the disease state of an individual) for use in the clinical setting, there may be a need to create a novel assay or diagnostic approach which improves the efficiency or detection quality over existing biomarker assays. In such a case, it is necessary for the newly developed technology to be compared and validated against the industry gold standard (e.g. ELISA) for that particular target (OIE 2013).

4.1.3.3 Cancer biomarker multiplexing

In terms of economic considerations, the delivery of mono-parametric cancer biomarker assays for widespread, routine medical uses are largely unrealistic. With this in mind, the multiplexing of cancer biomarkers is seen as a favourable option if biomarker assays are to form a cornerstone of personalised medicine in the future. Multiplexing has the potential to drastically reduce assay costs, resources needed and biological sample demands. There is an ongoing need to develop novel diagnostic assays which increase the depth of retrievable information from a biological sample whilst effectively dealing with the technical complexities which multiplex detection in complex fluids brings with it.

Companion diagnostic tests measuring single biomarkers have proven invaluable in measuring response or resistance to treatment, especially in cancers with well-defined molecular mechanisms of disease. However, in the cases where assessing response to treatment may rely on measuring more than one biomarker, the costs of running multiple different assays would limit their use. Looking toward a future of personalised medicine where multiple biomarkers will be assessed as a routine screening, systems must be developed which maximise the obtainable data from small biological sample volumes whilst keeping costs low (thus improving accessibility to cancer diagnostics).

Ideally, successful technological platforms for biomarker assessment will demonstrate adaptability and flexibility of function which will allow the rapid adoption of newly discovered biomarkers into the detection repertoire. A number of technological platforms have reached the market, which open the door to multiplexing in complex biological fluids. For example, the electro-chemiluminescent system developed by Meso-Scale

Discovery (Rockville, USA) has been demonstrated to quantify up to 40 biomarkers over a wide dynamic range in a 40ul blood sample (Stengelin et al. 2013). The BioPlex® 2200 system (Bio-Rad, USA) is a microbead-based multiplex assay which can assay up to 25 proteins at one time. Whilst these impressive systems have improved the state of available clinical diagnostic technologies, there is still room for improvement on aspects such as manufacturing material usage, adaptability to new cancer biomarker repertoire and costing. These examples underline the direction in which clinical diagnostics are moving (ie. away from mono-parametric cancer biomarker analysis) and so it is incumbent upon any emerging technological platforms to demonstrate their ability to detect and quantify multiple cancer biomarkers in complex biological samples, whilst demonstrating strong economic benefit for their use in clinical laboratories around the world.

In developing this nanowire platform towards multiplexed detection of cancer biomarkers in complex biological samples, it was first necessary to show that the nanowire platform could detect multiple proteins in isolation from each other (i.e., the different nanowire types were tested in isolation from each other). The nanowire assay was adapted for detection of EGFR and PSA detection, simply by changing the antibodies on the nanowire, as well as the accompanying complementary antibody (see section 2.3.2 for the antibody pair selections for EGFR and PSA biomarkers). In changing the antibody pairs (the capture antibody coupled to the nanowire surface and the detection antibody added during the assay process, refer to table 3.3 for an overview of the assay setup developed for ErbB2 detection), the rest of the nanowire assay components remained the same. This is important, as ultimately the three different nanowire batches will need to be integrated together to form a multiplex assay. It was a limitation of the current setup that each of the capture antibodies targeted against ErbB2, EGFR and PSA, were all tagged with the same fluorophore (AF488).

This meant that if the wires were mixed together, there was no way of telling them apart by their fluorescent signal. Future assay designs will build on this work by assigning a separate fluorophore to each capture antibody, thus allowing mixing of nanowire assays and therefore true multiplexing of biomarkers. Alternatively, a different sorting mode may be deployed which could combine magnetic detection of barcoded nanowires (for nanowire type identification) with optical detection of fluorescent detection antibodies, which would eliminate the need for a fluorophore on the capture antibody altogether. Identification and differentiation of nanowire groups (e.g. anti-ErbB2, Anti-EGFR, anti-PSA) based on magnetic signals would also facilitate multiplexing.

4.2 Results

4.2.1 Detection of spiked cancer biomarkers in complex biological media

4.2.1.1 Detection of spiked sErbB2 in blood plasma using the nanowire assay

Detection of sErbB2 in spiked complex biological solutions (relevant physiological sErbB2 concentration ranges from 15 – 150 ng/ml) was demonstrated using the nanowire assay. Known concentrations of sErbB2 were spiked into reconstituted blood plasma and measured using the nanowire assay (Figure 4.5). The results are presented as PE-A Median signal, measured in the PE channel in the cytometer. The data demonstrate that the PE fluorescent signal obtained from the developed anti-ErbB2 nanowire assay correlated positively with the sErbB2 standard curve. The assay can readily detect samples with higher sErbB2 concentrations (150 ng/ml and 100 ng/ml) and differentiate them from 0 ng/ml sErbB2 (Figure 4.5 (a)). The PE-A Median values in the 50 ng/ml and the 0 ng/ml were not significantly different enough to allow discrimination between the two concentration samples, however the intragroup variability was sufficiently low in a follow-up experiment (b) to allow discrimination of 50 ng/ml from 0 ng/ml sErbB2 and BSA (Bovine serum albumin, used as a control protein. It is commonly used in assay procedure as a blocking agent) control.

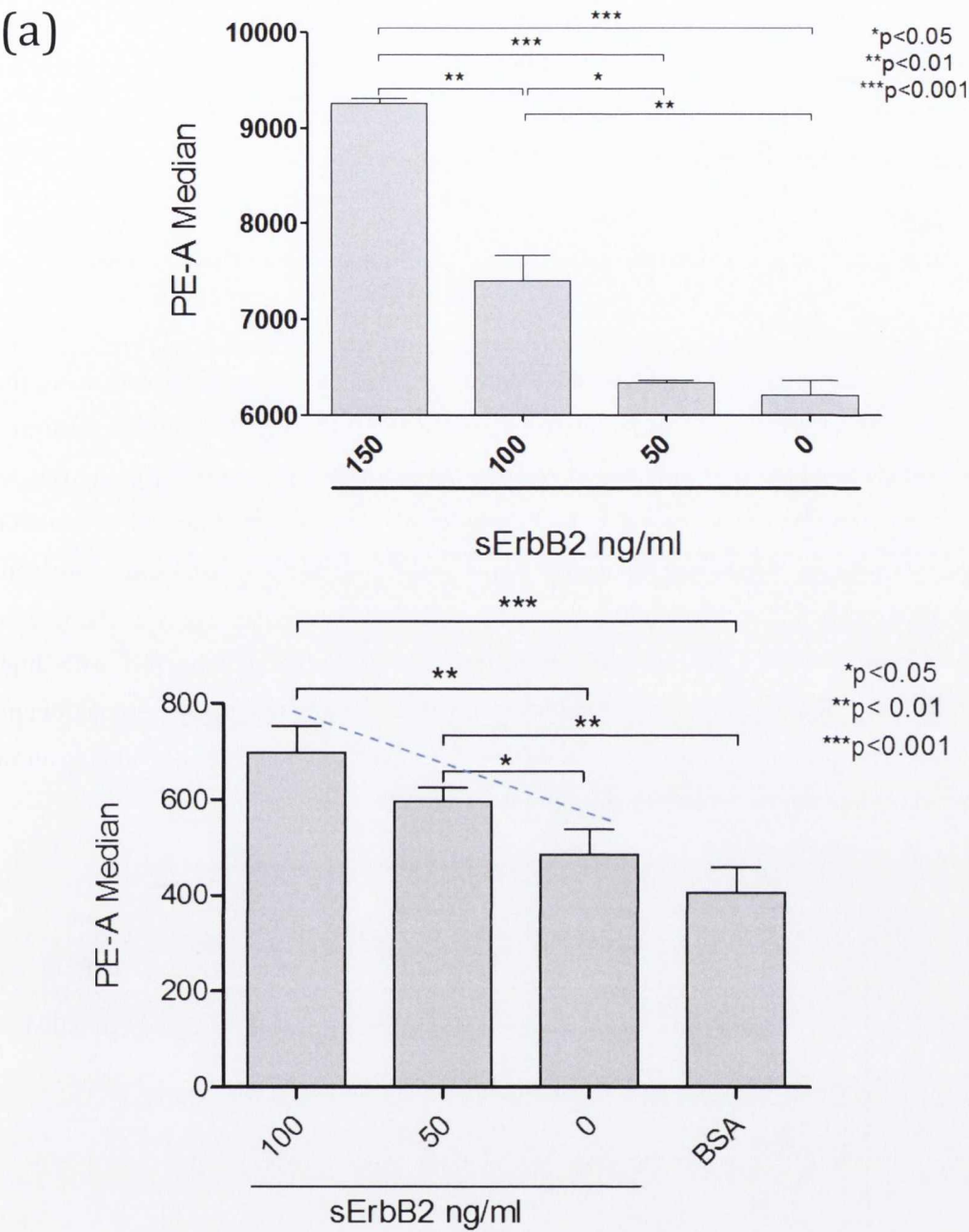


Figure 4.5 Detection of spiked sErbB2 in reconstituted plasma using the developed nanowire assay. The data is visualised as PE-A Median signal and expressed as mean $\pm$ SD. Results show that the PE-A Median signal is largest in samples containing high concentrations of sErbB2 and there is a positive correlation between sErbB2 concentration and PE-A Median signal. Statistical analysis: 1-way ANOVA followed by Tukey post test.

The data presented in figure 4.5 shows that fluorescent signals recorded from the nanowire assay correlated with the concentration of spiked sErbB2 in the complex biological samples, suggesting that the nanowire assay can detect different concentrations of sErbB2 in complex biological media. The intra-group variability in PE-A Median values was low enough so as to allow statistical discrimination of the sErbB2 samples from one

another. In addition, the linearity of the response over a large concentration range (0 – 100 ng/ml) was favourable (see the guide-to-the-eye (blue dashed line) annotated on figure 4.5 (b)), meaning the assay produced a linear response over a physiologically relevant concentration range.

4.2.1.2 Detection of spiked EGFR in blood plasma using nanowire assay

Detection of EGFR in spiked complex biological solutions was demonstrated using the developed nanowire assay. Known concentrations of EGFR were spiked into reconstituted blood plasma and assayed using the developed nanowire assay (Figure 4.6). The results are presented as the median value of fluorescence area for the fluorophore CF633, measured in the APC channel in the cytometer. The data demonstrate that the fluorescent signal obtained from the anti-EGFR nanowire assay correlated positively with the known EGFR standard curve (250 ng/ml, 150 ng/ml, 100 ng/ml and 0 ng/ml) with high significance. Although there was a smaller significance value between the 150 ng/ml and 100 ng/ml groups, the intragroup variability was sufficiently low in these groups to allow differentiation of the two concentration points.

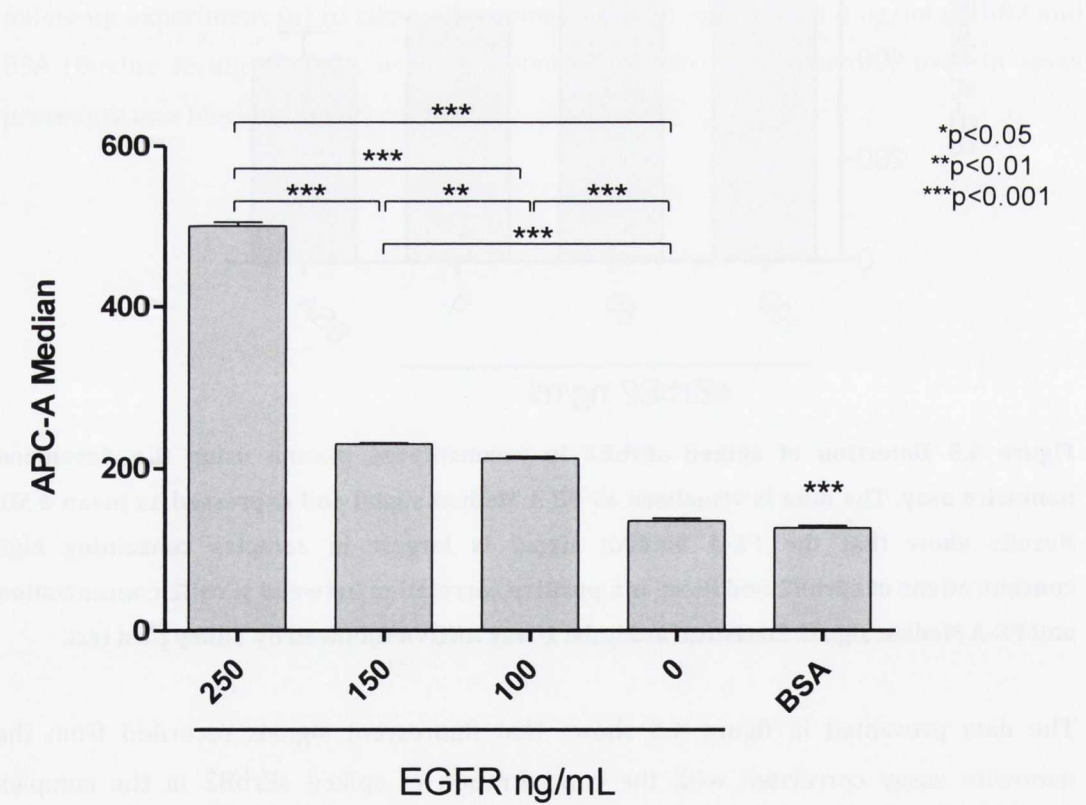


Figure 4.6 Detection of spiked EGFR in complex reconstituted plasma using the nanowire assay. The data is visualised as APC-A Median signal and expressed as mean $\pm$ SD. Data analysis shows a significant difference between all samples examined. For clarity purposes,

the significance stars above the BSA sample indicate a significance between 250 vs BSA, 150 vs BSA and 100 vs BSA groups. Statistical analysis: 1-way ANOVA followed by Tukey post test.

The data suggest that the nanowire assay is capable of detecting spiked EGFR in a complex biological sample. The intragroup variability in the EGFR assay is lower than that of the ErbB2 assay and this could be a consequence of using a detection antibody which is directly tagged with a fluorophore (CF633), compared to the sErbB2 assay which uses a fluorescent (PE) secondary antibody in order to indirectly tag the detection antibody. The data also shows that the presence of BSA (Bovine serum albumin was used as a control protein. It is commonly used in assay procedure as a blocking agent) in the complex sample did not lead to an increased PE-A Median signal using the nanowire assay.

4.2.1.3 Detection of spiked PSA in single analyte model using the nanowire assay

Detection of PSA was first demonstrated in a single analyte model using the developed anti-PSA nanowire assay. Known concentrations of PSA were spiked into PBS and assayed using the nanowire assay. The results are presented as the median value of fluorescence area for the fluorophore CF633, measured in the APC channel in the cytometer. The data shows that the assay could detect the highest concentration of PSA at 200 ng/ml and there was a significant difference in the fluorescent readout between the 200 ng/ml samples and the 0 ng/ml samples. However, the signal variation recorded in the 100 ng/ml and 50 ng/ml groups prevented the samples from being accurately measured and distinguished from any other sample (Figure 4.7). In order to test PSA in a complex biological sample, cell lysate from prostate cancer cell lines was assayed.

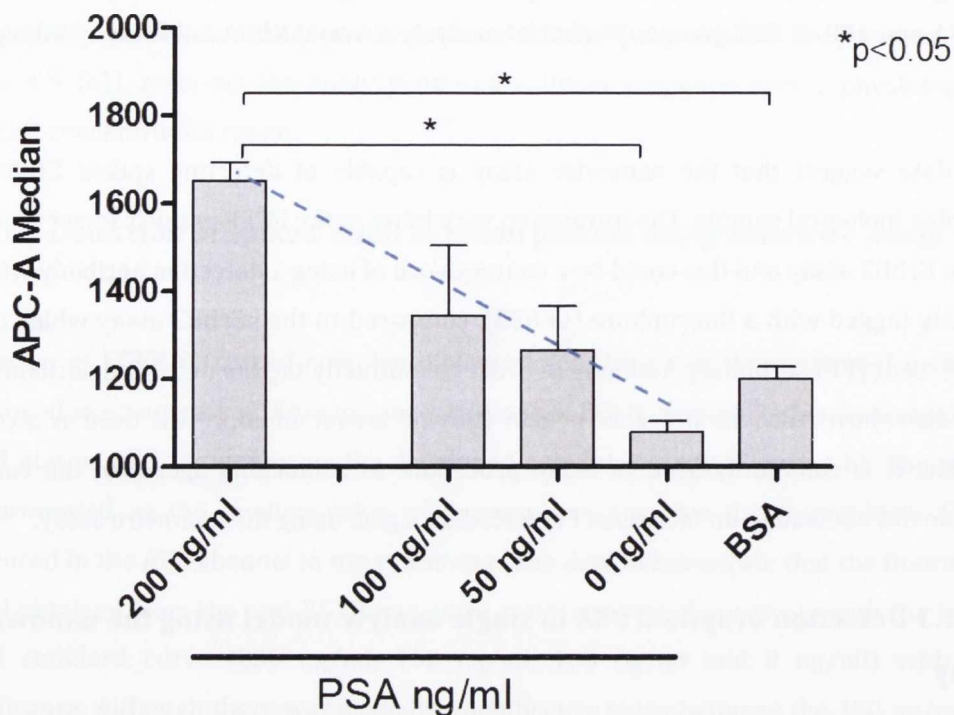


Figure 4.7 Detection of spiked PSA in single analyte sample using the developed nanowire system. The data is visualised as APC-A Median signal and expressed as mean $\pm$ SD. Data shows a significant difference between the median signal obtained from the 200 ng/ml PSA sample and both the 0 ng/ml and BSA non-specific target control samples ($p < 0.05$). The APC-A median values in the 100 ng/ml PSA and the 50 ng/ml PSA sample were not significantly different from each other. Statistical analysis: 1-way ANOVA followed by Tukey post test.

The data suggests that the nanowire assay for PSA can detect the presence of PSA in solution, but in this instance, it can only detect a high PSA concentration. This result may come as a consequence of testing the assay in a single analyte model. It is possible that with the testing of the anti-sErbB2 and anti-EGFR nanowire assays (4.2.1.1 & 4.2.1.2), the use of complex media helps to reduce non-specific binding events and as the above assay was carried out in a single analyte model, the blocking efficiency was reduced, resulting in greater non-specific binding and intragroup variation. Interestingly, the addition of a guide line (figure 4.7, dotted blue line) suggests that the nanowire assay is capable of returning a linear response across the concentration curve of PSA, which is similar to the linear response noted in the sErbB2 samples (figure 4.5). This is a positive trend for the nanowire assay and could be a considerable advantage in future multiplexed assays which are required to work over large concentration ranges.

4.2.1.4 Detection of PSA biomarker in cell lysates using the nanowire assay

The PSA concentration in the cell lysate of two prostate cancer derived cell lines was quantified using ELISA (Ray Biotech, USA). As before, the values obtained from the commercial PSA ELISA kit were normalised to the total protein content in the cell lysate as measured by Bradford assay, and displayed as PSA pg/μg total protein. The cell line LnCap was shown to contain a high concentration of PSA, whilst PC3 contained negligible levels of PSA. The two cell lysates were ideal for initial testing of the anti-PSA nanowire assay as both cell lines represented opposite ends of the spectrum in terms of PSA expression and LnCap and PC3 represented good PSA-positive and PSA-negative samples for testing, respectively. Additionally, testing the nanowire system using cell lysates offered an alternative complex biological solution for testing the nanowire assay in more realistic assay conditions, compared to the previously performed single analyte detection (section 4.2.1.3).

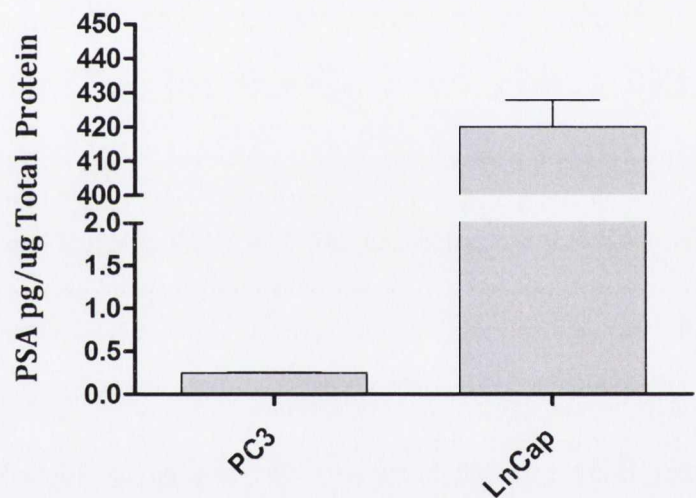


Figure 4.8 ELISA quantification of PSA in cancer cell line lysate. Data is expressed as mean PSA pg/μg Total Protein ± SEM (n=3). The data shows LnCap as a high expresser of PSA whilst PC3 shows very low levels of PSA expression. These cell lysates provide good PSA-negative (PC3) and PSA-positive (LnCap) complex biological samples for testing of the anti-PSA nanowire assay. Statistical analysis: Student T-test.

Detection of native PSA in PC3 and LnCap prostate cancer cell lysates (previously quantified using ELISA) was demonstrated using the nanowire assay (Figure 4.9). The results are presented as the median value of fluorescence area (APC-A Median) for the fluorophore AlexaFluor647, measured in the APC channel in the cytometer. The results show a significant difference (*p<0.05) in signal response between PC3 and LnCap, as

well as LnCap and BSA samples, and the intragroup variability in the PC3 and BSA samples was low.

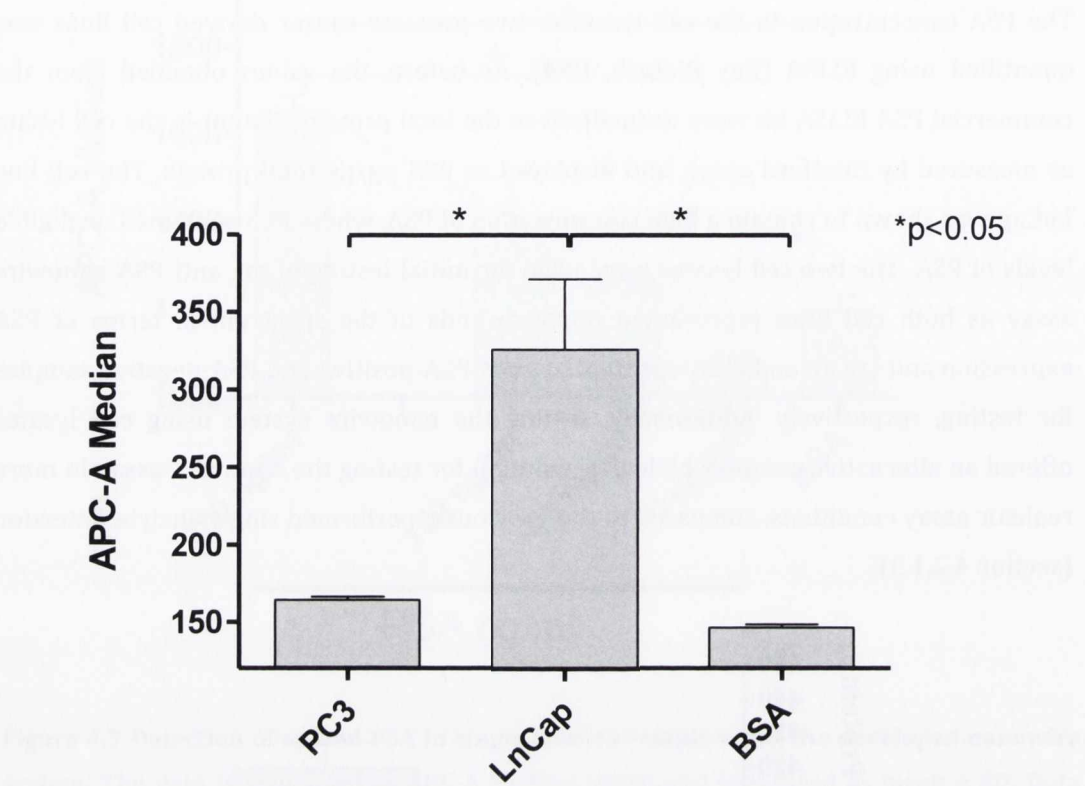


Figure 4.9 Detection of native PSA in prostate cancer cell lysates using the developed nanowire system. The data is visualised as APC-A Median signal and expressed as mean $\pm$ SD. Data shows a significant difference between the median signal obtained from the LnCap cell line and both the PC3 and BSA samples. Data is expressed as mean $\pm$ SD. Statistical analysis: 1-way ANOVA followed by Tukey post test.

The results suggest that the anti-PSA nanowire assay is capable of detecting native PSA in cell lysates. A low fluorescence signal (recorded as APC-A Median) was recorded in both the PC3 and BSA sample, and indeed both values are not significantly different from one another. There was a large fluorescent signal detected in the LnCap sample and this signal was significantly different ($*p<0.05$) from either the PC3 or BSA samples. The results indicate that the PSA detection antibody is attached to the nanowire following incubation in the LnCap lysate but not attached following incubation in the PC3 or BSA samples. This suggests that the anti-PSA nanowire assay can detect PSA in complex biological media such as cell lysates.

4.2.2 Detection of cancer biomarkers in clinical samples

Patient samples (blood for ErbB2 and EGFR, urine for PSA) were received for testing with the developing nanowire assay system. Samples containing between 200 – 500 µl each were received for each biomarker group (ErbB2, EGFR and PSA). Prior to receiving them, the concentrations of the respective biomarkers in these samples were assayed by our biotech industrial partner Progenika (Table 4.1). The samples were tested in triplicate with the nanowire assay using 30 µl sample.

Table 4.1. Biological samples used to test the nanowire system.

Biomarker					
ErbB2		EGFR		PSA	
Sample ID	ng/ml	Sample ID	ng/ml	Sample ID	ng/ml
ERBB2-S1	55.23	EGFR-S2	177.23	PSA-S4	1.12
ERBB2-S7	3.22	EGFR-S5	62.73	PSA-S5	436.28
ERBB2-S8	17.38	EGFR-S9	130.79	PSA-S10	69.33

In earlier experiments (see Section 3.2.11.2), although the nanosphere assay showed a larger relative signal difference between 25 ng/ml sErbB2 and 0 ng/ml sErbB2 samples compared to the nanowire assay, the latter provided a more linear response to the increasing sErbB2 concentrations. It is important to consider that while the nanosphere assay outperformed the nanowire assay over the initial 0-25 ng/ml range, the concentrations of sErbB2 in a clinical setting stretch beyond this concentration point. In clinical practise, a cut-off threshold of 15 ng/ml sErbB2 is used, above which point the patient is considered to have elevated levels of sErbB2. Indeed the concentration of sErbB2 measured in biological fluids can extend past 100 ng/ml sErbB2. For this reason, the linearity of the response of the nanowire assay was seen as favourable, as it stretched over a much wider range (covering a larger portion of the clinically relevant assay window) than the nanosphere assay. For this reason, the nanowire platform was used for testing in clinically realistic samples.

4.2.2.1 Detection of native ErbB2 cancer biomarker in blood using nanowire assay

The performance of the developed nanowire assay was tested in clinically realistic samples (blood) and the results were compared to those generated from independent ELISA measurements of the same samples. The results are displayed as the PE-A Median values of the nanowire assay on the left axis of the graph, and the corresponding mean sErbB2 concentration values as measured by ELISA on the right axis of the graph.

The data show that the results obtained from the anti-sErbB2 nanowire assay correlated with the data obtained from ELISA measurement of the same samples. The fluorescent signal observed in the low and medium sErbB2 samples correlated positively with the data from the ELISA, however there was considerable variability in the nanowire assay fluorescent readout in the high sErbB2 sample. The results suggest that the fluorescent signal measured from the nanowires, following incubation with high, medium and low sErbB2 clinical samples is proportional to the level of sErbB2 in each sample as measured by ELISA, particularly in the low and medium sErbB2 samples.

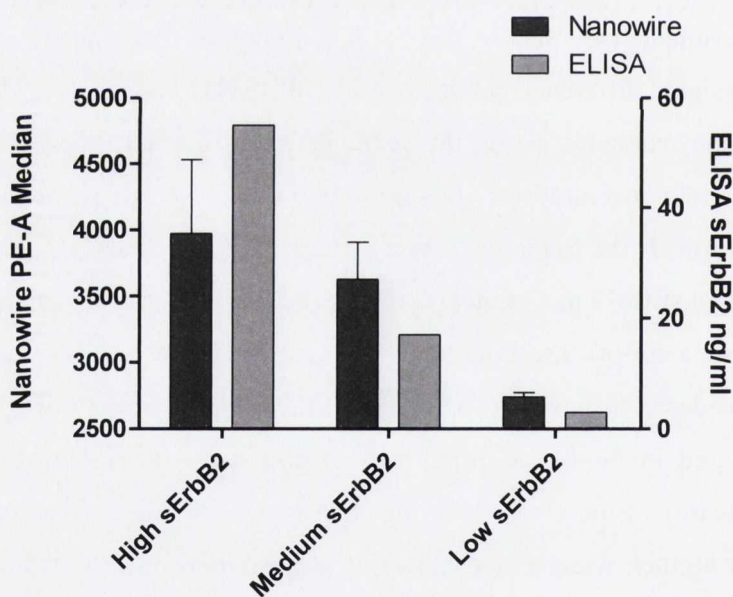


Figure 4.10 Comparison of native sErbB2 concentration in clinical samples as measured by anti-ErbB2 nanowire assay (left axis, dark grey bars) and conventional ELISA (right axis, light grey bars). The left axis displays the PE-A Median values (mean $\pm$ Std Dev) obtained by the nanowire assay whereas the right axis displays the sErbB2 ng/ml (mean values provided following independent testing of the samples) as determined by ELISA.

The variability observed in the sErbB2 assay above was not observed in the EGFR and PSA nanowire assays. The possible reasons for why this occurs in the sErbB2 assay are

discussed in section 4.3. Intragroup variability aside, the data provides evidence that the developed nanowire assay can detect native sErbB2 in clinical samples.

4.2.2.2 Detection of native EGFR cancer biomarker in blood using the nanowire assay

The performance of the anti-EGFR nanowire assay was tested in clinically relevant samples (blood) and the results were compared to those generated from ELISA quantification of the same samples. The results are displayed as the APC-A Median values of the nanowire assay on the left axis of the graph, and the corresponding mean EGFR concentration values as measured by ELISA on the right axis of the graph.

The results show that the fluorescent signal obtained from the anti-EGFR nanowire assay (APC-A Median) correlated with the data obtained from ELISA measurement of the same samples. The measured fluorescent signal was at its largest magnitude in the blood sample containing high EGFR, at a medium magnitude in the sample containing medium EGFR and low magnitude in the sample containing the smallest amount of EGFR. In addition, the intragroup variability in the EGFR nanowire assay was low. The results suggest that the fluorescent signal measured from the nanowires is proportional to the concentration of EGFR in each sample.

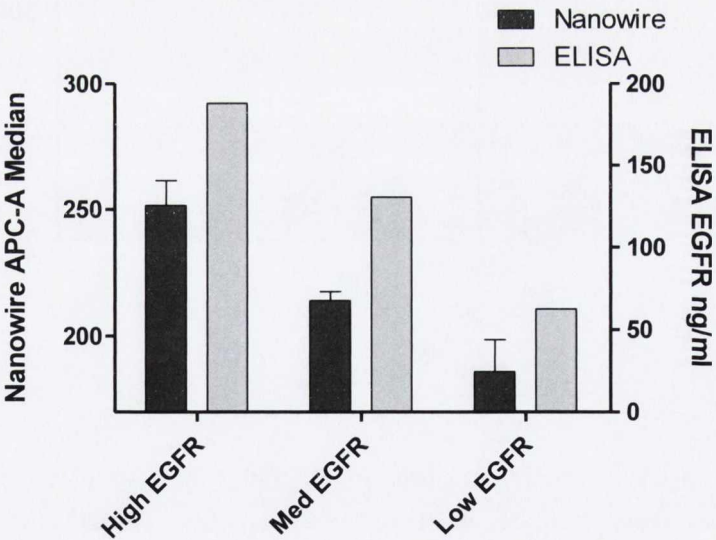


Figure 4.11 Comparison of native EGFR concentration in clinical samples as measured by the anti-EGFR nanowire assay (left axis, dark grey bars) and conventional ELISA (right axis, light grey bars). The left axis displays the APC-A Median values obtained by the nanowire assay whereas the right axis displays the EGFR ng/ml mean values as determined by ELISA.

The data obtained supports the suggestion that the developed anti-EGFR nanowire assay can detect native EGFR in complex biological samples such as blood plasma and that the signal response is proportional to the concentration of EGFR in the samples tested.

4.2.2.3 Detection of native PSA cancer biomarker in urine using the nanowire assay

The performance of the anti-PSA nanowire assay in detecting native PSA was tested in clinically relevant samples (urine) and the results were compared to those generated from ELISA quantification of the same samples. The results are displayed as the APC-A Median values of the nanowire assay on the left axis of the graph, and the corresponding mean PSA concentration values as measured by ELISA on the right axis of the graph. The results show that the magnitude of the fluorescent signal (from the CF633 fluorophore) recorded in each sample correlated positively with the concentration of PSA in the sample as measured by ELISA. The intragroup variability of the nanowire assay was very low.

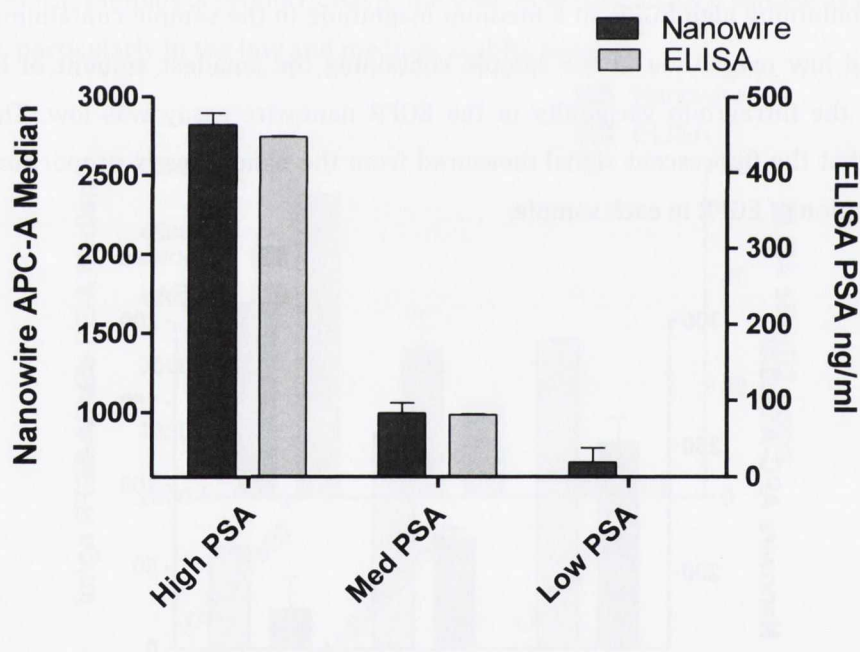


Figure 4.12 Comparison of native PSA concentration in clinical samples as measured by the anti-PSA nanowire assay (left axis, dark grey bars) and conventional ELISA (right axis, light grey bars). The left axis displays the APC-A Median values obtained by the nanowire assay whereas the right axis displays the PSA ng/ml mean values as determined by ELISA.

The results suggest that the fluorescent signal measured from the nanowires, following incubation with high, medium and low PSA samples is proportional to the level of PSA in each sample as measured by ELISA. The data obtained provides evidence that the

developed anti-PSA nanowire assay can detect native PSA in urine over a large concentration range (0 – 500 ng/ml).

A comparison was made between the nanowire assay and the ELISA in an attempt to compare the utility of each assay. The three assays (ErbB2, EGFR and PSA) were plotted, nanowire assay vs ELISA, in a dot plot and a best fit line applied to each (Figure 4.13). It was observed that for the ErbB2 assay, the nanowire assay appeared to saturate compared to the ELISA, whereas the EGFR and PSA assay performed equally as well as the ELISA. The observed saturation in the ErbB2 nanowire assay could be due to the suboptimal secondary antibody configuration used, as this saturation is not seen in the other two assays which use detection antibodies which are directly tagged with a fluorophore (vs ErbB2 which uses a fluorescent secondary detection antibody).

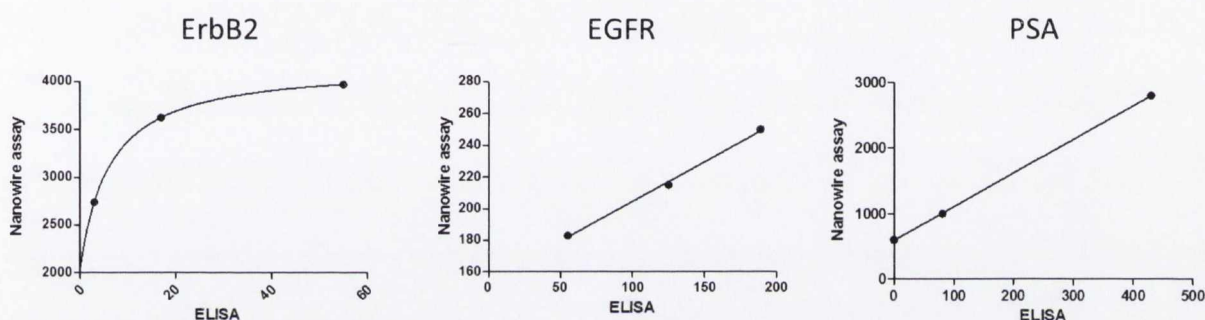


Figure 4.13. Comparison of nanowire assays vs ELISA for detection of ErbB2, EGFR and PSA in biological media. The nanowire data was plotted against the ELISA data gained from assay of biological samples. The data shows that for the ErbB2 assay, the nanowire assay appeared to saturate compared to the ELISA, whereas the EGFR and PSA assay performed equally as well as the ELISA in terms of signal response to increased biomarker concentration.

4.2.3 Comparison of theoretical reactive surface area of nanowire assay and ELISA as enabling technology platforms

In order to highlight one of the potential advantages of using a nanowire-based detection system over the conventional ELISA (or similar) systems, the theoretical reactive surface area of each approach was estimated and compared. The theoretical reactive surface was defined as the surface area on which a capture antibody could be immobilised (ie. an ELISA plate well or a defined number of nanowires), and thus the area on which an assay of a molecular target could take place.

For the ELISA well, the average area of the floor of a flat-bottomed well was used (based on well diameter of 6.4 mm) and calculations ignored the well wall area. For the nanowire assay, the theoretical reactive surface area was calculated by multiplying the theoretical surface area of one nanowire (assuming diameter of 150 nm and length of 10 μm) by the number of nanowires necessary per assay sample (taking the upper limit of 100,000 events favoured for robust statistical analysis, rather than the minimum event count of 10,000 employed by most commercial bead-based assays).

The theoretical calculation of reactive surface area for ELISA and nanowire was as follows:

ELISA well:

Well Diameter: 6.4 mm

Surface area circle = $\pi r^2 \rightarrow \pi (3.2)^2 = 32.17 \text{ mm}^2$

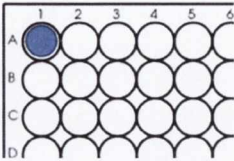
Nanowire:

Nanowire Diameter: 150 nm (or 0.00015 mm)

Nanowire Length: 10 μm (or 0.01 mm)

Surface area cylinder: $2 \pi r h + 2 \pi r^2 \rightarrow 2 \pi (0.000075) (0.01) + 2 \pi (0.000075)^2 = 0.00000475 \text{ mm}^2$

Table 4.2 Calculation of the theoretical reactive surface area of an ELISA and nanowire assay. Estimation of the unit surface area is made for each assay type and total surface area is obtained by multiplying the unit surface area by the number of units per sample.

	ELISA	Nanowire assay
Schematic		
Unit surface area	32.17 mm ²	0.00000475 mm ²
# Units per sample	1	100,000
Total surface area	32.17 mm ²	0.475 mm ²
ELISA:NW surface area ratio	68 : 1	

This calculation illustrates that whilst the nanowire assay is capable of detecting biomolecules in complex biological samples to a similar degree as an ELISA kit, it is able to do so using nearly 70 times less surface area per sample. It should be noted this calculation is based on recording 100,000 nanowire events, however, measuring 10,000 nanowire events would also be sufficient to allow statistical analysis of the data, which would reduce the necessary surface area by a factor of 10. This may have implications in reducing sample, antibody and reagent demands in future assay kits.

4.3 Discussion

In this results section, the proof-of-concept nanowire assay which was developed for detection of sErbB2 in a simple single analyte model was further progressed to be tested in complex biological samples (blood fractions and urine) and for two other biological targets, namely EGFR (Epidermal Growth Factor Receptor 1) and PSA (Prostate Specific Antigen). The silica nanowires were replaced by more uniform CoNi nanowires. The results demonstrate the functionality of the nanowire assay in detecting three separate cancer biomarkers in complex biological samples. The performance of the nanoprobe is put in context by comparison with pre-existing clinical assay standards.

4.3.1 Detection of spiked cancer biomarkers using the nanowire assay

Detection of spiked cancer biomarkers was carried out using the nanowire assay. In each case, the capture antibody was tagged with CF488 and the detection antibody/secondary antibody was tagged with PE (in the case of sErbB2) or CF633 (in the case of EGFR and PSA). Detection of spiked sErbB2 in blood serum was demonstrated using the nanowire assay. The data (Figure 4.5) demonstrates that the fluorescent signal obtained from the anti-ErbB2 nanowires correlated positively with the measured sErbB2 standard curve (150 ng/ml, 100 ng/ml and 0 ng/ml) with good confidence. Although in example (a) there was no significance value between the 50 ng/ml and 0 ng/ml groups, the intragroup variability was sufficiently low in a follow-up experiment (b) to allow differentiation of the two concentration points. These data are the first demonstration that this nanowire assay can detect a target cancer biomarker in a complex biological medium.

Detection of spiked EGFR in blood serum was also demonstrated using the anti-EGFR nanowire assay. In this case, the nanowire assay could successfully detect EGFR across the entire tested range (250 ng/ml, 150 ng/ml, 100 ng/ml and 0 ng/ml) with good statistical significance, based on the APC-A Median values obtained from the CF633 fluorophore which was attached to the EGFR detection antibody. There was a smaller significance value ($p < 0.01$) between the 150 ng/ml and 100 ng/ml groups, but all other relationships attained a significance value $***p < 0.001$. This data shows that the assay principles developed for the nanowire assay in sErbB2 detection in blood fractions can be translated (by substituting the capture and detection antibodies) to another cancer biomarker in the same fluid type. This illustrates the potential flexibility of the nanowire assay to be applied to the detection of multiple biomarkers in the same sample type.

Due to the fact that a realistic surrogate biological medium (ie. urine) was not readily available at time of testing, the detection of PSA in a simple single analyte system was demonstrated. The APC-A median values correlated with concentration of spiked PSA, and 200 ng/ml PSA could be differentiated from 0 ng/ml PSA and BSA non-specific target (* $p < 0.05$), however the variability in median signal in the 100 ng/ml and 50 ng/ml, whilst following a linear trend in line with the concentration curve, prevented the differentiation of samples at medium to low concentration levels. This detection discrepancy and degree of intragroup variability is in contrast to those observed when assaying ErbB2 and EGFR in complex biological media, and could be related to differences in the type of biological sample used (i.e. PBS vs blood serum). Indeed, when native PSA was assayed in biological samples (urine assayed in following section), the detection response of the nanowire assay is much more favourable than for the single analyte system.

The anti-PSA nanowire assay was used to detect PSA in cell lysates. The results show that the nanoprobe system registered a significantly higher APC-A Median value from the LnCap cell line, a value that was significantly different from both the PC3 cell lysate and the BSA non-specific target samples. The data is in line with the data gained from ELISA quantification of the respective cell lysates, and demonstrates that the nanoprobe system is capable of detecting native PSA in complex biological samples.

The presented data supports the conclusion that the nanowire assay can be used to detect several spiked target biomarkers in complex and simple biological media. By spiking the samples with known concentrations of target biomarkers, the detection of the analyte could be examined and the fluorescent response could be demonstrated. Additionally, the flexibility of the nanowire assay was highlighted by expansion of the biomarker repertoire to three different targets. Following on from these results, the nanowire-based assays were used to test native cancer biomarker levels in pre-validated complex clinical samples.

4.3.2 Detection of native cancer biomarker in complex biological samples using nanowire assay

Following the detection of three cancer biomarkers (sErbB2, EGFR and PSA) in cell lysates and spiked systems, the nanowire assay was used to detect native biomarker levels in realistic samples (ErbB2 & EGFR in blood serum and PSA in urine). Each of the clinical samples were selected to reflect clinically realistic high, medium and low cancer

biomarker levels, and the concentrations of each analyte were independently measured using ELISA prior to nanowire assay.

For ErbB2 samples, the developed nanowire assay was able to detect low and medium samples of native sErbB2 in blood serum with good correlation to the values obtained by the ELISA. In the high sErbB2 sample, although the nanowire assay yielded a PE-A Median value which was above that of the medium or low sErbB2 samples, the standard deviation in the replicates was larger than required. One possible reason for an increase in intragroup variability in the sErbB2 assay could be the use of a fluorescently-tagged secondary antibody in order to visualise the sErbB2 detection antibody on the nanowire surface, rather than using a directly-stained detection antibody (such as the CF633-stained detection antibodies used in the EGFR and PSA assays). It should also be noted that the quality of the fluorophore may also impact on the signal observed in the sErbB2 assay. The PE (phycoerythrin) fluorophore is known to have some spectral overlap with CF488 (the fluorophore used on the capture antibodies) and thus this interference, although compensated for electronically, may be affecting the fluorescent readout. Additionally, the stability of the PE fluorophore is not as good as the CF633 fluorophore, so it is possible that a deterioration in the PE signal occurred during the assay. From this, the data suggests that future assays use a directly stained detection antibody for more robust results.

That said, the nanowire assay returned a reasonably linear detection response in clinically relevant serum samples. In addition, the future use of a directly labelled detection antibody (rather than using the PE-labelled secondary antibody employed in this assay) like the ones employed for the EGFR and PSA assay, or the substitution of the PE fluorophore for a brighter fluorophore, may help to improve the accuracy and utility of the sErbB2 assay as it stands. As a proof-of-concept study, the nanowire assay demonstrated the potential to detect native sErbB2 in a complex clinical sample.

In EGFR-positive blood samples, the data shows that the developed nanowire assay was able to detect low, medium and high samples of native EGFR in biological samples with high confidence, yielding a reasonably robust response to the three different concentrations of EGFR, as measured by ELISA. There was a positive correlation with the ELISA results for this biomarker and the intra-sample variation was lower than those obtained in the ErbB2 assay. The observed intragroup variability noted in the EGFR and PSA data was much smaller than that observed in the ErbB2 data, and this may support a preference for using a directly-labelled fluorescent detection antibody, for reasons

mentioned above. The ability of this nanowire assay to detect native EGFR in blood serum highlights the flexibility of the nanowire assay to be applied to more than one cancer biomarker paradigm. The feasibility of this approach to be applied to a multiplex detection approach is also illustrated by the fact that the ErbB2 and EGFR nanowire assay used different detection fluorophores.

The nanowire PSA assay was able to detect low, medium and high samples of native PSA in biological samples with high confidence, and the fluorescent data correlated favourably to the PSA concentrations measured by ELISA. In addition, the standard deviation within the nanowire samples was very low. These results are in contrast to the previously shown results obtained from the assessment of PSA in a single analyte system. This observation illustrates how a change in assay media can have a considerable effect on assay outcome. It is possible that in the single analyte system, the inherent blocking effect contributed from the complex sample composition, was missing, and so led to increased non-specific binding. In the case of PSA assay in clinically relevant biological media, the assay appears to respond very well, detecting across a very large concentration range (500 ng/ml to 0 ng/ml). It is not surprising, perhaps, that the anti-PSA nanowire assay yielded the least intragroup variation of the three nanowire assays. Urine has less protein content in it than blood (Figure 4.1) so theoretically, there is lower competition for binding sites on the nanowire assay and perhaps less chance of non-specific binding events occurring. Interestingly, the detection of PSA in urine using the nanowire assay also demonstrates that the assay can be used across at least two complex biological media types (blood and urine). This may prove useful in helping to expand the clinical applicability of a developing nanowire assay.

The data presented shows that the nanowire assay could indeed be used to detect multiple native cancer biomarkers in complex biological solutions with signal values which were proportional to results obtained from the current gold standard in clinical assay. This proof-of-principle work, together with the improvements discussed in the following section, illustrate the potential clinical utility of this promising novel nanomaterial-based assay. The ability to perform a multiplexed assay was prevented by the use of the same fluorophore (CF488) to tag each of the different capture antibodies relating to each of the different targets (ErbB2, EGFR and PSA). This meant that during FACS analysis, it was not possible to distinguish the different nanowires from one another based on fluorescence. Future assays can circumvent this problem by using a different fluorophore tag for each of the capture antibodies or by detecting the nanowires by a different means, such as magnetic detection.

4.3.3 Comparison of theoretical reactive surface area of nanowire assay and ELISA as enabling technology platforms

One of the potential advantages of using a nanowire-based detection system over the conventional ELISA is that there is real potential to significantly reduce reagent and antibody demands. Due to the considerable monetary investment placed in research & development and production/validation of high-quality antibodies for the clinical diagnostics market, the cost of most antibody-based diagnostic assays are quite high. Therefore, any technology which could reduce sample and reagent demands whilst utilising fewer antibodies per assay may be a welcome addition to the in-vitro-diagnostics market.

Through comparison of the theoretical reactive surface area of an ELISA well (32.17 mm^2) and a sample batch of nanowires (0.475 mm^2), it is estimated that the nanowire assay could deliver data from a sample by using 70 times less reactive surface area, compared to conventional ELISA. This calculation has allowed for 100,000 nanowire events as the optimum event count for the assay. In fact, the industry standard event count per sample is 10,000, meaning that technically, if we assume no wire loss during the assay, that the nanowire system could deliver clinical sample data using 700 times less reactive surface area than conventional ELISA. This difference in reactive surface area size could have a knock-on effect on both sample demand and antibody requirements.

Although it is theoretically possible to reduce the area of the ELISA in an attempt to reduce cost, and some micro-well formats (the 1536-well micro plates) have a surface area of $\sim 1.86 \text{ mm}^2$, this is still an order of magnitude greater than what a nanowire platform can offer. In addition, there are no commercially available ErbB2, EGFR or PSA assays which use a format smaller than the industry standard 96-well plates as most assays aim to be compatible with the majority of laboratories which use the 96-well format. The amount of antibody used on the ELISA plate well varies between applications and it is titrated on a case by case basis, depending on factors such as the performance of the antibody and the cost of producing it.

4.3.4 Summary

In summation, through testing the performance of the nanowire assay in detecting three separate cancer biomarkers in a number of media, the data supports the conclusion that the nanowire assay is adaptable in terms of target specificity, flexible in terms of adaptability to different complex biological media and has the potential to reduce

antibody and reagent demands. Although there was sub-optimal intragroup variation in some of the samples tested, the work presented here provides strong evidence that the nanowire-based assays are capable of the detection of a number of cancer biomarkers in realistic clinical samples.

4.3.5 Concluding key-points

1. Demonstration of specific analyte detection in complex biological samples.
 - a. Detected ErbB2, EGFR and PSA using the nanowire assay in various biological samples.
 - b. Assay values correlated with ELISA measurements in clinical samples.

4.4 References

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Chapter 5

Conclusion

5.1 Conclusion

The development of new methods to assess cancer biomarker load in body fluid fractions will inevitably help to improve the quality of treatment which cancer patients receive. Nanomaterial-based assays provide one avenue to improve clinical diagnosis of cancer biomarkers with respect to sensitivity, sample and reagent demands, cost and multiplexing capabilities. The option to functionalise NPs with ligands has facilitated the introduction of nanomaterials to clinical diagnostic applications. Although currently, nanomaterial-based technologies suffer from limitations such as batch-to-batch reproducibility, it is expected that with constantly improving understanding of nanoparticle synthesis and functionalisation, the advantages of nanomaterial-based diagnostics will be fully realisable.

5.1.1 Building the nanoprobe assay: Validating the antibody-nanomaterial functionalisation approach

In this body of work, due to the fact that our knowledge of handling and use of nanomaterials is constantly evolving, it was first necessary to investigate a selection of different analytical approaches which could be used to qualitatively and quantitatively validate antibody-nanomaterial conjugates in terms of (a) confirming the presence of antibody on the nanomaterial surface, (b) assessing whether the antibody coverage was evenly distributed on the nanomaterial (c) calculating the proportion of the nanomaterial population which were sufficiently functionalised and (d) tracking the stability of the nanowire-antibody conjugates following functionalisation. A range of approaches were trialled and nanomaterials of similar composition but differing geometries were used in order to demonstrate that even nanoparticle geometry had a direct impact on the suitability of one analytical approach over another.

Confocal microscopy proved most useful in terms of qualitative assessment of antibody-nanomaterial conjugates, and this was made possible by the use of fluorescently-tagged antibodies. Although the fluorescent yield could be quantified for individual wires, the approach was not ideal for large population-based assessment of nanomaterial functionalisation. AFM (facilitating the topographical mapping of antibodies on the nanomaterial surface) and Helium ion microscopy (providing ultra-high resolution imaging of the nanomaterial surfaces) also provided supportive data to the qualitative assessment of individual wires, but the labour intensive nature of both approaches meant that they would be unfavourable for routine, large-scale quality control assessment of functionalised nanomaterials.

Approaches used to assess nanomaterial populations yielded more useful data in terms of assessing the overall success and uniformity of nanomaterial functionalisation and tracking the stability of the bio-conjugates over time. Of these analytical approaches, flow cytometry and nanoparticle tracking analysis (NTA) gave the most useful data. NTA was only suitable for analysis of spherical particles, and flow cytometry was favoured for analysis of nanowire populations. Gel electrophoretic and spectroscopic approaches were also useful in assessing spherical nanoparticle functionalisation, however, they were not suitable for yielding quantitative data on nanomaterial functionalisation. Realistically, the quality of the data obtained by gel electrophoretic and spectroscopic approaches, in terms of the depth of information, was not ideal when compared to that of flow cytometric or NTA analysis which could also give data on individual particles within a population.

In all, the utilisation of a range of complementary analytical approaches provided support for the conclusion that nanomaterial functionalisation with antibody was successful, reliable, quantifiable and stable. The generated data not only provides a satisfactory result in an academic sense, but provides a useful technical knowledge-base which could prove useful for the translation of nanowire technology to the industrial scale.

5.1.2. Testing the initial functionality of the nanoprobe assay

Following the validation of nanowire functionalisation, the ability of the nanomaterial assay to detect a specific protein target was tested. Demonstrating sErbB2 detection using the nanowire-based sandwich assay was an important first step in validating the basic functionality of the assay. The nanowire platform could preferentially detect sErbB2 over a BSA control and this provided the first evidence of the functionality of the antibody-conjugated nanoprobe. Following on from that, the complexity of the assay was increased from a single-fluorophore assay (just using a fluorescent detection antibody) to a dual-fluorophore assay (using both a fluorescent capture antibody, attached to the nanowire surface, and a fluorescent detection antibody) in order to improve the visibility of nanowires in the flow cytometer. Testing the ability of this assay setup to detect spiked sErbB2, it was shown that the fluorescent readout (in terms of Phycoerythrin (PE) fluorescent intensity) from the detection antibody as measured by flow cytometry, was in proportion to the amount of spiked sErbB2 present in a single analyte model system.

The system was improved in terms of assay procedure and component selection (for example, through comparison of candidate fluorophores) in readiness for testing in complex biological samples. The FITC-tag on the capture antibody (BMS120FI) which was used in the

initial sErbB2 detection experiments, proved to be suboptimal for allowing the visualisation of all the nanowire events in a flow cytometry setting, and was ultimately affecting the sensitivity potential of the nanowire assay. A systematic, dual-approach comparison of this fluorophore (FITC) with two other fluorophores (AlexaFluor488 and CF488) of similar fluorescent emission spectra clearly showed that FITC was not suitable for use in the nanowire assay. Subsequent substitution of FITC with CF488 lead to an improvement in nanowire visibility in a flow cytometric system and ultimately improved the quality of the nanowire assay.

The cancer biomarker repertoire was expanded in order to illustrate the versatility of function of the nanowire platform. Anti-ErbB2, anti-EGFR and anti-PSA CoNi nanowires were developed and validated and their ability to detect the target molecules in complex samples was investigated. Using a flow cytometric approach, it was possible to correlate the fluorescent output of the detection antibody with the presence and concentration of cancer biomarkers, both in simple systems and in complex biological samples. Data gained from cell lysate, spiked biological samples and native clinical samples demonstrated that the CoNi nanowires were capable of biomarker detection in complex media with comparable responses to that of ELISA. The results demonstrate the feasibility of using a nanowire-based system for flow cytometric assay of cancer biomarkers in blood and urine samples.

5.1.3 Developing new ways to observe nanomaterials in flow cytometric systems

In addition to the flow cytometric approaches developed for assessment of nanomaterial functionalisation, flow cytometry was also used to interrogate non-fluorescently tagged AgNW populations of varying lengths. Some nanomaterials, such as those made from Ag, pose a challenge to fluorescence-based detection systems as they strongly absorb many of the spectral emission wavelengths of common fluorophores. However, their ease of manufacture and uniformity make them an attractive source for possible incorporation into diagnostic systems. Consequently, an analytical approach which can demonstrate fluorescent-label-free detection and size discrimination of AgNWs may prove a useful tool for furthering their incorporation into other multimodal nanomaterial-based diagnostic systems. Additionally, the knowledge gained from studying the length-dependent optical scatter properties of AgNWs can ultimately be translated to the study of nanowires of different materials which share geometric similarities but do not absorb spectral emission wavelengths of fluorophores to the same extent (such as CoNi NWs).

Interrogation of silver nanowires in this novel manner allowed some very interesting observations to be made on the length-dependent light scatter properties of nanowires in a

sheath flow environment. Principally, the results supported the conclusion that wires over a certain length (in this case above 26 μm median length) would be more suitable for integration into future flow cytometric setup. It is thought that longer nanowires are more likely to become axially restricted in the laminar flow setup of the flow cytometer and so their enhanced alignment, perpendicular to the interrogation beam, results in a stronger side-scatter width response. Furthermore, the novel analytical approach developed to visualise the wires allowed a correlation to be made between nanowire length and the slope relationship between side-scatter height and side-scatter area. This work may contribute to a new understanding of the unusual light scatter signatures of nanowires and other anisotropic particles and further facilitate the application of these materials for clinical diagnostic and industrial applications.

In addition to the investigations into nanowire optical signals using a commercially available flow cytometer (BD FACS Canto), a custom-built RDS-CLX system was also used to detect nano/microspheres in a focused flow cell. Both detection systems were tested using a nano/microsphere size ladder (100 – 3200 nm). Measuring the inherent (non-fluorescence based) light scatter properties of the spheres, the commercial flow cytometer struggled to discriminate spheres of small diameters (100 – 280 nm) whilst the RDS-CLX system was able to clearly identify and discriminate individual sphere populations in the same size range with much better resolution than the cytometer. This increase in sensitivity of optical signal detection shown by the developed RDS-CLX system may provide an advantage to future diagnostic assays where reliable identification and discrimination of nanospheres of different sizes would allow multiplexing of analytes without the need to use dedicated fluorescent tags (resulting in a reduction in assay complexity).

The RDS-CLX system was also used to detect fluorescently-tagged CoNi nanowires and was successfully able to detect the fluorescently-tagged nanowires in a high throughput manner. Interestingly, analysis of the fluorescent peaks associated with the nanowires using the FITC width parameter gave the clearest discrimination of the nanowire populations from background signals, adding further support to earlier findings on the Ag nanowires, that the width parameter is most suitable for viewing nanowire signatures in a flow setup. The observations indicate that the RDS-CLX fluidics handling system, flow cell, optical components and acquisition software were successfully integrated and capable of the handling nanowires in a high-throughput manner. It is possible to further modify the RDS-CLX system to include sensors for other modalities such as magnetic detection systems, should they become available. Future studies may facilitate the integration of additional detection modalities, which may serve to increase the utility and applicability of the novel system to clinical uses.

5.1.4 Highlighting the advantages of nanowires in future diagnostic assays

Given the results obtained during the course of these studies, it is possible to highlight the potential advantages of the use of nanowires in future diagnostic assays.

Firstly, the incorporation of nanowires into diagnostic kits brings the strong possibility for reducing reagent demands, due to the large inherent surface-to-volume ratio of nanowires, in comparison to the existing ELISA diagnostic platforms. In particular, any assays which reduce the need for antibody (often the most expensive part of an assay kit) whilst still giving a measurable output, may reduce production costs and consequently, the cost of the diagnostic to the patient. The AFM approach was initially shown as a way to assess antibody coverage on the nanowire, providing confirmation that the antibody is distributed on the nanowire surface. Following on from that, theoretical calculations comparing the reactive surface area between the nanowire assay and ELISA estimate that the nanowire assay uses nearly 70 times less reactive surface area, whilst still being able to detect native biomarkers levels at the ng/ml concentration similar to ELISA measurements. The reduction in required surface area to perform the task (biomarker detection) in the case of the nanowire, has a knock-on effect on reagent and sample demands, and may also reduce assay reaction time. This observation highlights one facet of the potential advantages of using nanowires in diagnostic assays.

Second, the use of magnetic nanowires (CoNi) in this assay setup provides the opportunity to expand the assay to a multimodal approach which incorporates magnetic separation (during assay steps) and detection (during analysis) approaches. This brings with it the potential to further simplify diagnostic assays by removing reliance on a singular mode (i.e. fluorescence-based detection) for detection of the nanowires in a flow environment. The CoNi nanowires used in this assay development were found to be highly uniform, readily functionalised with antibody, reliably stable in solution, and performed well in detection of biological analytes. These characteristics, coupled with their magnetic properties could make them promising candidates for incorporation into multimodal diagnostic approaches.

Another interesting observed aspect of the use of nanowires is their tendency to produce a linear fluorescent signal response curve in proportion to the amount of analyte present, when compared to spherical particles. This appears to be the case especially over large concentration ranges (for example 0-250 ng/ml) which makes it ideal for testing of physiologically relevant biomarker concentrations. This linear response (see section 4.2.1.1

and 4.2.1.3) is a favourable characteristic as it simplifies the production and analysis of a calibration curve and would result in a more accurate quantification of analytes in diagnostic assays. By highlighting the apparent advantages of nanowires in a diagnostic assay, based on the presented observations above, nanowires should certainly be considered as an enabling technology for the improvement of diagnostic assays in the future.

The platform is not currently re-useable, but potentially, the collection of nanowires after assay could allow them to be recycled for use in future assays. However, the cost of retrieving, recycling and repurposing nanowires might not be an economically feasible option in the long run, given that nanowire fabrication is relatively easy to upscale, and they can be produced on a mass scale.

5.1.5 Safety considerations with developing nanomaterial assays

A vital consideration in the translation of a new nanomaterial-based diagnostics to market is that of safety. As our knowledge of the rules governing nanomaterial interactions with living systems grow, we are beginning to understand that material composition, geometry, exposure duration and concentration, among other factors, all contribute to whether a nanomaterial is considered toxic or tolerated by the body. It is incumbent on us to treat these materials with care and to have proper processes in place for the safe management of these materials when they are in use.

Although this author was not directly involved in the toxicity studies related to the nanomaterials used in the presented work (this work, instead was carried out by collaborators), this author has contributed to studies which assess the biocompatibility of nanomaterials in cell based systems (See Appendix 3 - 5). One study involved the investigation of AgNWs in cell lines and the study found that AgNWs were well tolerated in the two cell lines tested and this suggests that AgNWs could form part of a safe diagnostic platform in future. Quantum dots (QDs) have also been investigated as diagnostic probes for cell studies (See Appendix 4 & 5) and while QD-based probes were shown to have desirable qualities in terms of biomarker detection sensitivity, their potential toxicity to living cells means that they require tight regulation and disposal protocols when in commercial and medical use. The safety profiles of silica nanoparticles are probably the most investigated worldwide, and silica nanoparticles are generally well tolerated by living cells and they do not come with the same toxicity issues as QDs. Whichever the material, it is essential that the safety understanding of nanomaterials develops in tandem with the understanding of the potential applications of these materials, as their safety profile will ultimately determine the successfulness of nanomaterials as key enabling technologies for future diagnostic assays.

5.1.6 Concluding discussion

In conclusion, based on the results gained during the completion of this body of work, the use of nanowire based biological assays in future medical diagnostic settings remains a real possibility.

The results show that it is possible to effectively functionalise nanowires to make them useful for detecting biological targets. Most, if not all, of the approaches used to assess the quality of the nanowires, and then the nanowire-antibody conjugates, are available in mainstream industry, and the results presented in this thesis show which approaches are more fit-for-purpose in this case. The fact that the analysis of the nanowires did not require heavily customised machinery means that the analysis of nanowire-antibody conjugates as part of routine quality control is relatively attainable in any well-equipped lab or manufacturing facility, and thus makes the adoption of the developed technology relatively easy. The confirmation that the antibody was stably attached to the nanowire over a fixed time period (1 month) and anecdotally stable for over 6 months, means that the developed assay is compatible with the usage demands associated with existing commercially available assays. This again works in favour of the developed platform in terms of translatability to market.

Although initial work has been done on the assay optimisation for the nanowire-based assay, it is fair to say that further testing in this respect may need to be carried out. There may be other assay buffers which further improve the background and non-specific binding events during an assay, but this is really based on trial and error and dependent on knowledge of and access to a range of buffer solutions. This work has shown that the concentration of functionalised nanowires in a solution could be estimated, however, it would be useful to conduct a more in-depth comparison of a number of other approaches for this purpose, to see if the concentration estimates hold true. In saying that, the developed approaches provide a good basis for further developing a method of accurately measuring nanowire concentration in a solution.

In terms of biomarker detection, the results shown in the thesis are very encouraging. Not only could the nanowire assay detect biomarker targets in spiked samples, but also in complex biological fluids such as blood and urine. That considered, the nanowire assay does require more testing in a much greater number of samples in order to establish the robustness and reliability of such a system. Realistically, this kind of testing is required to be done in a more stringently controlled environment than that available in the current academic laboratory set-up.

The results presented in this thesis also showed that the nanowires could be fluorescently tagged, could be recorded in a high throughput manner based on the attached fluorescent tags, and that data from fluorescently-tagged detection antibodies could be simultaneously detected. This approach was successful in demonstrating the ability of the platform to adapt to different biomarker requirements and this adaptability could be exploited in future to develop a truly multiplex biomarker detection system, either by changing the fluorophore colour on the nanowire or by using a different mode of detection (for example, magnetic detection of barcoded metallic/non-metallic nanowires) in order to capture data from the nanowire in a high throughput manner. The latter requires a technological development which was outside the scope of the presented work, but which could be incorporated into the detection system at a later date. This opens the possibility of truly multi-modal detection systems which simplify data capture and assay interpretation.

In short, the proof-of-concept device has been successfully developed and demonstrated, and the technology now requires transfer to an industry setting capable of developing and testing the platform further.

In terms of justifying whether the nanowire-based assay is worthy of translation to a clinical setting, the potential advantage of using this system must be highlighted. It is necessary therefore to identify where the potential strengths of the system lie over existing technologies (ie. ELISA and other immunoassays). The potential strengths of the nanowire system appear to exist in three camps. Firstly, it is the potential to differentiate individual nanowires from each other in a high throughput manner (either by fluorescent-based sorting or magnetic detection of barcoded metallic/non-metallic nanowires) which makes the multiplexing potential of this technology a real possibility. This would have the effect to lower the cost of running biological assays whilst increasing the data output from a single biological sample. As we move toward an era of personalised medicine, there is an unstoppable shift towards measuring and analysing multiple biomarkers, and so this technology is well-placed to be adapted to a multiplex purpose. Secondly, confirmation of antibody immobilisation on the assay (ie. nanowire) surface can be measured in real-time during assay analysis, by measuring the fluorescent signal from each nanowire. This is a real advantage, as this is not possible with existing ELISA setups, where we must assume that the antibody is evenly and faithfully distributed on the micro-well plate. Thirdly, the reduced antibody requirements of the nanowire assay are another potential asset worth translating to future clinical diagnostics. In current biological assays, the main cost of the assay is anchored in the antibody used in the kit. Buffers and other reagents are relatively cheap compared to the value and cost placed on antibodies within an assay kit, especially if the kit is approved

for use in a clinical (rather than research laboratory) setting. The nanowire assay demonstrated in this thesis returned results comparable to ELISA readings, but did so using ~70 times less reactive surface. If we assume that using less assay reactive surface translates to less antibody demands and therefore usage, the nanowire platform could be an ideal way of reducing costs associated with the most expensive part of current biological assays, the antibody.

In terms of assessing improved performance, maybe we need to start to look beyond improving functional performance of an assay. In practise, current assays are already capable of detecting miniscule amounts of target protein in biological samples, and so striving to improve on this is not really going to return much advancement in our use of diagnostic assays. In a world where market forces and access to wealth largely dictates whether people can access medical services, maybe improved assay performance should take into account the cost of production and use of the assay. In this respect, as demonstrated in this thesis for just one type of platform, nanotechnology has the potential to provide cost-effective solutions for clinical diagnostics which harness the power of existing assays and enhance their functionality, reduce their cost and improve access to healthcare for the world's ever-growing population.

5.1.7 Concluding key-points

1. Achieved characterisation, functionalisation and validation of nanomaterial-antibody conjugates.
 - a. Confirmed the presence of antibody on the nanomaterial surface.
 - b. Assessed antibody distributed across the nanomaterial.
 - c. Estimate the proportion of the nanomaterial population which was sufficiently functionalised.
 - d. Track the stability of the nanowire-antibody conjugates following functionalisation.
2. Demonstration of specific analyte detection in single analyte and complex biological samples.
 - a. Detected ErbB2, EGFR and PSA using the nanowire assay in various biological samples.
 - b. Assay values correlated with ELISA measurements in clinical samples.

3. Development of novel approaches towards optimal nanowire visualisation in a flow cytometric system.
 - a. Identified SSC-W as the most appropriate parameter for viewing nanowire length-associated responses.
 - b. Developed a novel gating strategy to analyse nanowire signatures in flow cytometry.
4. Demonstration of a custom-made acquisition system for nanomaterial assays.
 - a. Improved sensitivity in detection of nanomaterials over conventional flow cytometers.
 - b. Achieved detection of nanospheres and functionalised nanowires using the setup.

In conclusion, the work presented in this thesis summarises the progress towards development of a nanomaterial platform for fluorescence-based flow cytometric detection of soluble cancer biomarkers in clinical samples. The work has highlighted the characterisation, functionalisation and validation of nanomaterial-antibody conjugates, and progress has been made towards the development of nanomaterial-based assay approaches for flow cytometric systems for detection of soluble proteins in complex biological samples. Data analysis approaches for high-throughput nanowire interrogation have been developed and the work has also demonstrated the improved detection sensitivity of a custom-made acquisition system over existing commercial flow cytometers. It is hoped that this progress can be furthered in future to deliver a nanomaterial platform for biomarker detection worthy of use in a clinical setting.

Appendix 1

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**THE ADELAIDE & MEATH
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Professor Yuri Volkov
Department of Clinical Medicine
Institute for Molecular Medicine
Trinity Centre for Health Sciences
St. James's Hospital
James Street
Dublin 8

May 20th 2010

REC reference: 2010/05/02

(Please quote REC reference and EudraCT number on all correspondence)

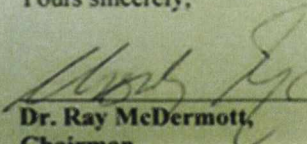
**Re: Nanotechnological Toolkits for Multimodal Disease Diagnostics and
Treatment Monitoring (NAMDIATREAM).**

Dear Professor Volkov,

The SJH / AMNCH Research Ethics Committee, having reviewed the above application at its meeting held on May 12th 2010, decided to give ethical approval to this proposed study subject to the following:

- o The Committee asks that the Patient Information Sheet & Consent Form be rewritten

Yours sincerely,



Dr. Ray McDermott,
Chairman,
SJH/AMNCH Research Ethics Committee

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Dublin 8

July 15th 2010

REC reference: 2010/27/01

(Please quote REC reference and EudraCT number on all correspondence)

Re: NAMDIATREAM

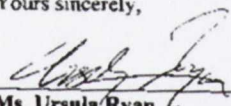
Dear Professor Volkov

Thank you for your email communication dated June 17th 2010 which you sent in response to the Committee's letter of May 20th 2010.

The Chairman of the Committee has reviewed your response and the revised Patient Information Sheet & Consent Form has given ethical approval on behalf of the Committee.

Full ethical approval is now in place for this study.

Yours sincerely,



Ms. Ursula Ryan
Secretary
SJH/AMNCH Research Ethics Committee

Appendix 2



Molecular Probes™
Invitrogen detection technologies

Catalog Number	F-13838
Name	Flow cytometry Size Calibration Kit *nonfluorescent microspheres*
Appearance	white suspension
Medium	distilled water, 0.05% Tween® 20, 2 mM sodium azide
Lot	413597

Technical Data from the Supplier of the Unstained Microspheres

Density of polystyrene

Component A - C 1.055 g/cm³

Component D - F 1.060 g/cm³

Component	Concentration ¹ particles/mL	Actual particle size
A	6.0×10^7	$1.1 \mu\text{m} \pm 0.035 \mu\text{m}$
B	3.0×10^7	$2.0 \mu\text{m} \pm 0.039 \mu\text{m}$
C	2.0×10^7	$4.2 \mu\text{m} \pm 0.19 \mu\text{m}$
D	2.0×10^7	$5.9 \mu\text{m} \pm 0.05 \mu\text{m}$
E	2.0×10^7	$9.9 \mu\text{m} \pm 0.11 \mu\text{m}$
F	2.0×10^7	$15.4 \mu\text{m} \pm 0.18 \mu\text{m}$

1. Calculated from size and percent solids.