

Lab-on-a-chip based immunosensor principles and technologies for the detection of cardiac biomarkers: a review

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This review examines the current state of the art lab-on-a-chip and microfluidic based biosensor technologies used in the detection of cardiac biomarkers. The determination and quantification of blood based, cardiac biomarkers are crucial in the triage and management of a range of cardiac related conditions, where time delay has a major impact on short and longer-term outcomes of a patient. The design and manufacturing of biomarker detection systems are multi-disciplinary in nature and require researchers to have knowledge of both life sciences and engineering for the full potential of this field to be realised. This review will therefore provide a comprehensive overview of chip based immunosensing technology as applied to cardiac biomarker detection, while discussing the potential suitability and limitations of each configuration for incorporation within a clinical diagnostics device suitable for point-of-care applications.

1 Introduction

1.1 Motivation for cardiac biomarker detection

Cardiovascular disease (CVD) is one of the most prevalent medical conditions of modern times and accounts for approximately half of all the deaths within the western world.¹ CVD related conditions cost the European Union approximately 192 billion Euros per year, representing a major financial burden on

clinical resources.² One of the most immediately life threatening forms of acute coronary syndromes (ACS) is myocardial infarction (MI), which is defined as the necrosis of cardiac myocytes following prolonged ischemia. As MI causes irreversible damage to the heart, a patient suspected of MI must be diagnosed as quickly, efficiently and comprehensively as possible using information obtained from the readings of the electrocardiogram (ECG) and the measurement of cardiac specific biomarkers within the patient's blood. For hospital admissions related to ACS up to 50–70% of patients demonstrate normal or ambiguous ECG reading.^{3,4} The assessment of cardiac marker elevation is therefore required for the clinician to make a truly

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informed decision on a suitable course of treatment. Elevated levels of such markers can give indicative information on the type of ACS experienced by a patient, the approximate time of first incidence of the attack and, for certain markers, the location of the damaged cells.

Clinicians routinely perform tests of cardiac markers to enhance MI rule out, with results predominantly ascertained by an on-site specialist clinical chemistry department. Internationally recognised guidelines for cardiac marker diagnosis recommend a turn-around-time (TAT) of less than 60 min once a patient is admitted to the hospital. This guideline has however proven to be problematic with less than 25% of hospitals attaining this recommended target.⁵ The failure to achieve this TAT increases the time taken for a patient to receive an initial course of treatment. The immobilisation of the patient within the clinic, besides being discomforting, further increases the financial burden to health providers. What would be desirable is for a clinician to perform a rapid and absolute measurement of the cardiac marker concentrations at the time of first contact. Such a tool could enhance patient care, increase the speed and efficacy of cardiac treatment and diagnosis by a clinician, resulting in major cost benefits to the health service sector.

To meet this clinical demand a handful of point-of-care (POC) immunosensing instruments have recently become commercially available as shown in Table 1. These devices can provide results within 5–20 min yielding comparable sensitivities to the larger clinical instrumentation. They have, however, yet to gain wider acceptance within the clinical community. The cost restraints imposed on hospitals, a lack of clear integration into existing clinical equipment and routine practises, the requirement to perform independent large scale evaluation studies to establish biomarker clinical cut-off concentrations for a given local population, are all factors slowing down the penetration of these devices into hospitals.

Currently there are numerous cardiac related biomarkers that are considered efficacious towards the diagnosis of the various forms of CVD. The measurements of a specific marker can provide valuable insight into the specificity of the condition, location of myocardial damage, the initial time of on-set/progression and can potentially replace alternative diagnostic strategies such as echocardiograms. Table 2 summarises many of the primary clinically utilised cardiac biomarkers, highlighting the major clinically relevant parameters, such as their efficiency for CVD diagnostics and their respective cut-off values. The typical clinical practise is to take several measurements of a patient's blood cardiac marker concentration such that elevation profiles can be established to monitor the progression of the presenting condition relative to a given course of treatment.

Due to the complexity of CVD, no single biomarker can be considered a definitive indicator encompassing all possible conditions. For example, Troponin I (cTnI), is considered the 'gold standard' in terms of diagnosing MI, owing to its presence only resulting from direct damage of myocardium. However, this biomarker is hindered due to relatively long duration, 4–6 h, of initial elevation relative to the presenting physical symptoms.^{4,9} Conversely, myoglobin elevation is observed within a relatively short time period, 1–3 h,⁴ however, lacks the specificity of troponin as it is found in both skeletal and cardiac muscular

tissue and may be elevated following skeletal specific muscular damage.

1.2 Introduction to immunoassays

Immunoassays have become the gold-standard technique in clinical diagnostics for determining cardiac and other clinically relevant biomarkers. All current biosensor formats aimed at cardiac biomarker detection use some form of immunoassay, involving the binding of a specific antibody to a unique site on a target biomarker (antigen). Antibodies are proteins found within the blood/bodily fluids of vertebrates and are produced by white blood cells. For immunoassay purposes, a monomer antibody is utilised which comprises of a 'Y' shaped structure that contains two antigen binding sites on the upper tips of the protein. The recognition site of a target antigen by a specific antibody binding site is known as the epitope. Antigens contain numerous epitopes but antibody binding to a unique epitope is a highly specific interaction, providing the capacity for antibody/antigen recognition in solutions containing many different biomolecules. For this reason the immunoassay provides a highly repeatable and highly specific reaction format, suitable for a range of target biomarker based biosensing applications. Antibodies are produced as monoclonal and polyclonal varieties, with monoclonal antibodies binding to a single epitope and polyclonal antibodies being capable of binding to multiple epitopes. For this reason the selection of antibodies is a non-trivial issue when optimising a given assay format and, in many instances, multiple antibodies, both monoclonal and polyclonal, may be utilised in trail reaction in order to maximise reaction kinetics and to determine a suitable immunoassay specific to a given antigen target. In general, the use of monoclonal antibodies increases the reaction specificity but potentially at the expense of the reaction sensitivity. Additionally, the cross-reactive nature of polyclonal antibodies, in particular when attempting multiplexed assays, may lead to undesired antibody/antigen binding, hindering the overall specificity of an immunoassay as compared to monoclonal antibodies.

In recent studies, molecules known as aptamers have been utilised as antibody alternatives, functioning in a similar fashion with molecular recognition of the target biomarker.^{15–17} Aptamers and their use in biosensors for biomarker based detection is an emerging field and have less prominence over the use of antibodies due to complexities related to their design for unique target antigen epitope recognition and the optimisation of reaction kinetics. Aptamers will not be discussed any further in this review.

The immunoassay process has been exploited in a variety of novel sensor formats, resulting in a wealth of immunosensor technologies. Advancements have been achieved in both the chemical processes surrounding signal generation and the use of high sensitivity equipment and techniques. In broad terms, an immunosensor operates by relating the immunoassay event as a generated optical or electrochemical signal that is related to the concentration of captured biomarkers. An immunosensor must have then the capacity to efficiently capture the target antigen and relay this information as a detectable signal. In that respect, the so called sandwich structure, shown in Fig. 1, is the most widely used configuration for the capture of target antigens. This

Table 1 Performance and detection methods of current commercial POC devices utilised for the detection of cardiac markers

Device	Detected cardiac markers	Detection range/limit of detection	Assay time	Dimensions	Detection method
AQT90 (Radiometer)	(1) Troponin I (2) CKMB (3) Myoglobin (4) NT-proBNP (5) C Reactive Protein (6) BnCG (7) D-dimer	(1) 0.01–50 $\mu\text{g L}^{-1}$ (2) 2–500 $\mu\text{g L}^{-1}$ (3) 20–900 $\mu\text{g L}^{-1}$ (4) 12–35 000 ng L^{-1} (5) 5–500 mg L^{-1} (6) 2–5000 IU L^{-1} (7) 80–100 000 $\mu\text{g L}^{-1}$	Tests done in parallel. 30 tests per hour	Benchtop instrument	Time-resolved fluorescence detection
Triage cardiac panel (Biosite) ⁷	(1) Troponin I (2) Myoglobin (3) CKMB	Quantitative measurement above clinical baseline concentrations. Precise sensitivity unknown.	~15 min	8.5'' D $\times$ 6.25'' W $\times$ 2.75'' H. Small benchtop POC device	Fluorescence intensity detection
Cobas h232 (Roche Diagnostics Ltd)	(1) Troponin I (2) Myoglobin (3) D-dimer (4) proBNP (5) CKMB	(1) 0.1–2 ng mL^{-1} (2) 30–700 ng mL^{-1} (3) 0.1–4 $\mu\text{g mL}^{-1}$ (4) 60–3000 pg mL^{-1} (5) 1–40 $\mu\text{g mL}^{-1}$	~8–12 min	275 $\times$ 102 $\times$ 55 mm. Handheld device suitable for POC applications	Fluorescence intensity detection
Cardiac STATus (Nanogen)	(1) Troponin I (2) Myoglobin (3) CKMB	Qualitative measurement of either above or below a pre-determined clinical cut-off value.	$\leq$ 15 min	Handheld test strip (similar to home pregnancy test kit)	Chemiluminometric detection
i-STAT (Abbott Point of Care)	(1) Troponin I (2) CKMB (3) BNP	(1) 0–50 ng mL^{-1} (2) Unknown (3) Unknown	>2 min (as stated on website)	Handheld device suitable for POC application	Electrochemical detection (amperometric)
Cardiac Reader System (Roche Diagnostics Ltd)	(1) Troponin T (2) Myoglobin (3) NT-proBNP (4) D-dimer (5) CKMB	(1) 0.1–2.0 ng mL^{-1} (2) 30–700 ng mL^{-1} (3) 60–3000 pg mL^{-1} (4) 0.1–4.0 $\mu\text{g mL}^{-1}$ (5) 1.0–25 ng mL^{-1}	~8–12 min	250 $\times$ 200 $\times$ 95 mm, small benchtop device, total weight of 1.81 kg	Fluorescence intensity detection
RAMP 3.2 (Response Biomedical Corporation)	(1) Troponin I (2) CKMB (3) Myoglobin (4) NT-proBNP	(1) <10% (at 0.2 ng mL^{-1}) (2) <10% (at 7.2 ng mL^{-1}) (3) <10% (at 100.0 ng mL^{-1}) (4) Unknown	Only stated as within minutes.	27 $\times$ 25 $\times$ 15 cm, benchtop device, total weight 2.1 kg	Fluorescence intensity detection
Alpha Dx (First Medical Inc.)	(1) Troponin I (2) CKMB (3) Myoglobin	Lower detection limits of: (1) 0.09 $\mu\text{g L}^{-1}$ (2) 0.5 $\mu\text{g L}^{-1}$ (3) 5 $\mu\text{g L}^{-1}$	<20 min	Small benchtop device	Fluorescence intensity detection
Evidence Investigator biochip array (Randox laboratories LTD) ⁸	(1) Troponin I (2) CKMB (3) Myoglobin (4) CA III (5) FABP (6) GPBB	(1) 0.66 $\mu\text{g L}^{-1}$ (2) 1.23 $\mu\text{g L}^{-1}$ (3) 1.35 $\mu\text{g L}^{-1}$ (4) 2 $\mu\text{g L}^{-1}$ (5) $\geq$ 1.2 $\mu\text{g L}^{-1}$ (6) $\geq$ 1.2 $\mu\text{g L}^{-1}$	>1 h	Microarray, chip based device	Chemiluminometric detection

Table 2 Summary of prominent clinically utilised cardiac biomarkers for the diagnosis of various forms of CVD

Cardiac biomarker	Clinical cut-off levels	Initial elevation above clinical cut-off (h)	Duration till peak elevation (h)	Duration of elevation	CVD indicator type	Specificity (low, medium, high)
Troponin I ^{1,4,9}	0.01–0.1 ng ml ^{-1a}	4–6	18–24	4–7 days	Detection of MI and tool for risk stratification	High: specifically increased following myocardial necrosis
Troponin T ^{1,4,9,10}	0.05–0.1 ng ml ^{-1a}	3–4	18–24	10–14 days	Detection of MI and tool for risk stratification	High: specifically increased following myocardial necrosis
Myoglobin ^{1,4,11,12,51}	70–200 ng ml ⁻¹	1–3	6–12	12–24 h	Early detection of MI and reperfusion	Low: released following skeletal muscle injury, rapid clearance following necrosis and perceived increases due to compromised renal function
CK-MB mass assay ^{4,12}	10 U L ^{-1a}	3–4	12–24	24–36 h	Early detection of MI	Medium: released following skeletal muscle injury
h-FABP ^{4,13,14}	6 ng ml ^{-1a}	1–3	6–8	24–36 h	Early detection of MI	Low: released following skeletal muscle injury, rapid clearance following necrosis and perceived increases due to compromised renal function
C Reactive Protein ^{1,4}	<1 µg ml ⁻¹ low risk, 1–3 µg ml ⁻¹ intermediate risk >3–15 µg ml ⁻¹ high risk. Still no definitive consensus	No clinical consensus ~1–3	No clinical consensus	No clinical consensus	CVD related inflammatory response based biomarker.	High: a large body of evidence to suggest a direct link between elevations above clinical cut-offs and recurrent ischemic events
NT-proBNP ^{1,4}	0.25–2 ng ml ⁻¹ . No clinical consensus	No clinical consensus	No clinical consensus	No clinical consensus	Ventricular overload and indicator of stresses due to ischemia or necrosis	High: well established link between elevated biomarker levels and diagnostics of acute heart failure

^a Recommended as the 99th percentile (mean ± 3 standard deviations) in a pre-determined 'reference' population, with a CV of <10%. Above this value is considered abnormal.

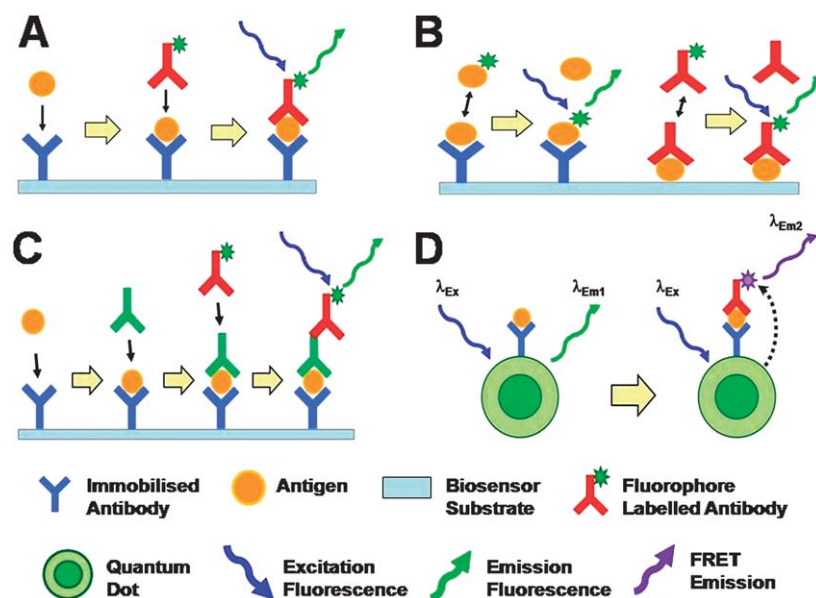


Fig. 1 A diagram illustrating four possible immunoassay binding configurations suitable for biosensing applications. Shown are the progressive reaction steps leading to the final binding structures for: (A) sandwich structure formation using a fluorophore labelled secondary antibody, (B) competitive style immunoassays using labelled antibodies/antigens, (C) extended sandwich structure formation using a fluorophore labelled tertiary antibody and (D) sandwich structure formation on a quantum dot (microparticle) surface using a secondary FRET paired fluorophore.

immunoassay consists of a sensor/reaction substrate functionalised with immobilised primary reaction antibodies, complementary to the target antigen. The immobilisation of the antibody is performed primarily as a means of separating the sample target biomarker during the phases of sample introduction and evacuation. It also allows the spatial addressing of the detection/sensor area. A solution containing the biomarker of interest is introduced over the antibodies, and, following an incubation period, the sensor area is washed of any free molecules. Upon completion of the stage, binding is expected to have occurred between the antibodies and antigens. Subsequently, a second solution is introduced containing secondary labelled antibodies, complementary to a remaining free epitope on the now captured antigen. A second incubation is performed, which allows the labelled antibodies to bind to the remaining available site on the bound biomarker. This is immediately followed by a second washing phase. In this configuration the target biomarker is essentially 'sandwiched' between the two reaction antibodies. This described immunoassay format is by no means the only possible reaction configuration and alternative binding formats for antigen identification are illustrated in Fig. 1 and represent the primary biosensors formats currently demonstrated within the literature.

The generation of an immunosensor signal resulting from antigen capture is predominantly realised by some type of label on a secondary reaction antibody. There is much freedom over the type of antibody label and selection is dependent on the specific detection methodology. This includes fluorescent labels,^{18–25,28–33,35,38–40,72,73} enzymes (for catalysis of a colour changing/redox reactions),^{43–47,61,80–82,97,98,100–102} paramagnetic particles (inductance/magnetic field based measurement)^{112–114,116,117} and metallic colloids as SERS probes.^{78,79,83,84} Various research groups have also

investigated the possibility of label free immunoassay biosensors,^{58,59,61,94–96,105,106,119–125,127–130,132,133,136–146} which offer many advantages, such as the omission of a second reaction incubation phase, a reduction in complexity of both the acquisition of the biosensor signal and the overall reaction chemistry, a reduced reaction TAT and cost per assay as antibodies and fluorophores contribute to the bulk of the cost for single assay. The immediate benefits of label free biosensors may however be offset by the additional and unique challenges introduced by their implementation, as explained later.

Cardiac biomarker detection is a field of medical diagnostics that could benefit immeasurably from the combined application of lab-on-a-chip (LoaC), biosensor and microfluidic based technology, to create a new generation of portable and fully automated POC test kits. The multi-disciplinary nature of such technologies requires that researchers have an appreciation of both the biological and engineering aspects of such devices for the full potential of this field to be realised. Many excellent reviews have examined singular facets of the total technology required for cardiac biomarker detection, discussing the clinically applicability of the various known cardiac biomarkers,^{1,4,6,10,11,147} various biosensing technologies^{17,26,51,93,99,105,126,134,148–152} and microfluidic/LoaC technology for clinical and general bioanalysis.^{153–159} The main aim of this review is to examine the current state of the art LoaC/microfluidic based biosensor technology used in the detection of cardiac biomarkers and to discuss each demonstrated technologies merits and limitations towards incorporation into a complete POC diagnostics system. Central to such a technology is the biosensing component, which predominantly utilises some form of immunoassay and requires various reaction specific methodologies of reagent delivery and incubation. The evaluated biosensor signal generally dictates the final structure and portability of a resulting diagnostics

system, given the current constraints of manufacturable and commercially obtainable instrumentation. The immunosensing technology examined in this review can equally be applied to the detection of alternative bioanalytes, for example cancer and environmental biomarkers, however, given the pressing urgency associated with CVD diagnostics and the envisaged major real world impact, cardiac biomarker detection is prioritised in this review. Certain reviewed sensor configurations may not have been demonstrated directly with cardiac biomarker detection. Despite this, for promising chip based detection formats, we briefly describe the respective technique and leave the challenge open for researchers to evaluate such biosensors further with cardiac biomarkers. Given the wide variety of interest in immunoassay based biosensors by numerous research groups, this review does not pretend to be exhaustive, but aims to inform the reader of the prominent biosensor configurations in a tutorial style approach.

2 Optical detection methodologies

Within the biological research community, the most common way of recognising an immunoassay event is by the optical detection of either fluorescent labels or a perceived colour change due to enzymatic action. Optical based techniques are currently regarded as the 'gold standard', allowing detection sensitivities down to ng–pg mL⁻¹ and have been the subject of many outstanding reviews.^{25,51,75,148,151–153} Optical detection encapsulates quite a broad spectrum of biosensor techniques and can be divided into the categories of intensity,^{18–42,45–49} surface plasmon^{58–76} and Raman^{77–92} based biosensor signals. Each specific technique has inherent advantages and disadvantages and a detection modality is selected based on the experimental application. For POC applications, the selection is based on a trade-off between cost, sensitivity, complexity, reproducibility, device component portability and power requirements.

2.1 Optical intensity based immunosensors

Optical intensity based measurements are arguably one of the simplest forms of biosensor signals to produce and analyse. The optical signal produced can be either fluorescent or luminescent. Fluorescent signals are produced by the excitation of a suitable label (fluorophore, quantum dot, *etc.*) bound on the surface of the sensor. Luminescent signals are produced following an enzymatic reaction (horseradish peroxidase, *etc.*) of a lumino-genic substrate solution (luminol, luciferin derivatives, *etc.*) or activation following a highly energetic electron transfer process occurring due to the application of a potential at an electrode surface (electrochemiluminescence, ECL). Typically, signal production, acquisition and analysis can be achieved using simple and inexpensive apparatus, with far less excitation and detection stringency than alternative optical based methods such as SERS and SPR. General intensity measurements are by far the most examined optical detection method with respect to immunoassay sensors, requiring just a suitable excitation source such as a laser diode or LED, simple light collection apparatus (lenses, optical fibres, *etc.*) and a reasonably sensitive photodetector (CCD, photomultiplier, *etc.*), all of which are commercially available. Furthermore, luminometric based biosensors

can be particularly advantageous as the luminescent signal only requires the use of optical collection and detection apparatus, reducing design complexity, costs and overall power requirements.

2.1.1 Fluorescence. Fluorescence intensity measurements have been demonstrated in two distinct assay formats, either using a single fluorescent probe or by the use of two Förster resonance energy transfer (FRET) paired fluorophores. FRET based immunosensors shall be discussed in greater detail later in this review and so this section focuses on immunosensors utilising a single fluorophore label. Such biosensors generally require reaction washing phases to allow for the removal of any unbound antibody labels. The need for such a removal increases the operational demands and the time for a single assay. The selection of a suitable probe is arbitrary, but is generally based on the suitability of the label for the particular sensor application and on factors such as spectral output, quantum efficiency, cost, *etc.* Fluorescence intensity based biosensors typically require the use of a fluorescence microscope and photodetector such as a CCD or photomultiplier, in order to adequately image the small sensor area, which is typically of the order of μm^2 .^{21,22,24,25,28,31,38}

The detection of a target biomarker is performed by the formation of the antibody/antigen sandwich structure. In this configuration the primary antibody is immobilised onto the sensor substrate and the secondary antibody is labelled with the detection fluorophore. Any unbound labelled secondary antibodies are then removed from the sensor area. The excitation of the sensor area leads to fluorescence emission from the fluorophore labels. The detected fluorescence signal directly relates to the concentration of captured biomarkers within the sandwich structure and allows for quantitative determination of the biomarker concentration.

As this type of methodology is such a well examined technique in both biological and engineering research communities, current research has focused less on the refinement of the biosensor elements but more on the methodologies for multiplexed reactions^{18–21} and the integration of immunosensors into chip based microfluidic platforms.^{23–25} Such developments are the logical progression towards a complete POC device and modern devices are enabling capabilities such as serum sample filtration and non-mechanical microfluidic delivery, all of which are conducted on chip.^{23–25} These devices are also capable of detecting ng mL⁻¹ concentrations of specific biomarkers within minutes from the time of sample introduction into the microfluidic chip. In work by Jönsson *et al.*,²³ a lateral flow polymer chip was demonstrated for the detection of the cardiac biomarker C Reactive Protein (CRP). The device is impressive in its simplicity, with the whole device comprising of only a simple sample introduction area, a fluidic channel and a wicking area, as shown in Fig. 2A. The biosensor portion of the chip consists of reaction antibodies immobilised as a line across the fluidic channel (detection zone), which act to capture the CRP from spiked serum samples as they flowed over the sensor area. Following migration of the serum sample to the wicking zone, a solution containing Alexa 647 labelled secondary antibodies is placed into the sample zone and migrates across the detection zone, leading to antibody/antigen sandwich structure formation. The concentration of captured

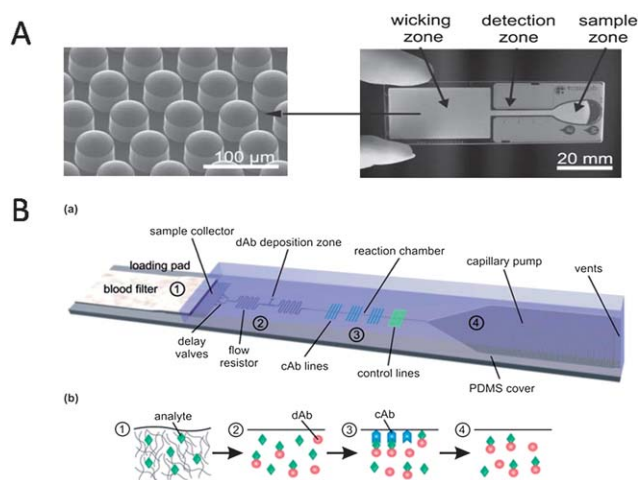


Fig. 2 (A) Lab-on-a-chip cardiac marker detection device as demonstrated by Jönsson *et al.* 2008.²³ Fluorescence intensity measurements are performed by the imaging of Alexa 647 labelled antibodies immobilised within the detection zone using an in-house prototype line illuminating fluorescence scanner. Copyright © The Royal Society of Chemistry 2008. (B) Diagram illustrating the lab-on-a-chip cardiac marker detection device as demonstrated by Gervais *et al.* 2009.²⁵ Fluorescence intensity measurements are performed by imaging of Alexa 647 labelled antibodies immobilised within the reaction chamber (3) using a fluorescence microscope. Copyright © The Royal Society of Chemistry 2009.

CRP is found to be proportional to the fluorescence emitted from the Alexa fluorophores following excitation and allows for a detection limit of 2.6 ng mL^{-1} .

In the work by Gervais *et al.*,²⁵ an impressive microfluidic device was demonstrated, capable of performing a full automated immunoassay through the simple addition of a test serum sample onto a loading pad to initiate the device. The immunoassay reagents are housed on chip using flow restriction channels and a capillary pump directs the sample through the channel containing the reagents and on to the biosensor region, as shown in Fig. 2B. This design significantly reduces operator demands per assay as there is no requirement for washing phases, allowing for assays to be completed within minutes. The chip also benefits from having no power requirements, further enhancing the desirability of this design for POC applications. The immuno-sensor section comprises of immobilised antibodies deposited within the main fluidic channel, on which the biomarker is captured and sandwich structure is formed with an Alexa 647 labelled secondary antibody. Quantification of the biomarker concentration is made relative to the detected fluorescence of the labels. Demonstrations of the device have yielded detection sensitivities below ng mL^{-1} for the analysis of serum samples containing various concentrations of the cardiac biomarker CRP.

Organic fluorescent molecules traditionally used for fluorescence detection based immunoassays have been replaced by some researchers with semiconductor nanoparticles known as quantum dots (QDs). Organic fluorescent dyes, although reasonably effective in their use, suffer from poor photostability, photobleaching, self-quenching with high labelling concentrations, decreased quantum yield when conjugated to biomolecules and a broad excitation/emission spectrum.^{28–30} QDs have

demonstrated reasonably high large quantum yields, a strong resistance to chemical and photo degradation, large absorption cross sections spanning a broad range of excitation wavelengths, tuneable emission profiles and narrow emission profiles. They can also be manufactured with coatings to enhance their biocompatibility and solution solubility.^{28–30,34} In the work by Su *et al.*³⁰ a QD immunosensor was developed for the detection of *E. coli* 0157:H7, using magnetic bead based separation in place of the typical washing phases. In their work the comparison signal produced by the organic dye fluorescein isothiocyanate was at least 100 times less sensitive than a QD fluorophore. The major limitations to the use of quantum dots are their cost, which is the primary reason why fluorophores are utilised in preference to QDs, particularly for high throughput applications. A further potential issue in the use of QDs for bioassays are their size (typically 10–50 nm), which is comparable in size or larger than the antibody it is labelling. Following binding, the larger resulting molecule suffers a reduced diffusion rate in comparison to a free antibody/fluorophore labelled antibody, resulting in degraded immunoassay sensitivity. The size of the QD is also crucial when designing microfluidic channel structures within which the QD is to be transported, too small a channel may actually lead to blocking.

The application of QDs to immunosensor based technology has seen considerable research interest,^{27,28,30–38} however, demonstrations of their targeted usage towards the detection of cardiac markers are limited.²⁹ QDs can also be used as an antibody immobilisation substrate in FRET based immunoassays.^{29,37,38} The potential simultaneous excitation of QDs with independent emission profiles from a single excitation source makes them also a clear contender for multiplexed immunoassays reactions.^{28,34,36}

FRET is a distance dependent, non-radiative transfer (dipole–dipole coupling) of excitation energy between a donor and an acceptor chromophore. When two such chromophores are within the Förster radius from one another and following excitation of the donor, the excitation energy can be transferred to the acceptor chromophores excited state, leading to the fluorescence emission. The FRET signal is therefore predominately based on the fluorescence intensity contribution due only to the acceptor fluorescence. FRET will only occur provided that there is sufficient spectral overlap of the donor emission and the acceptor excitation spectra. The acceptor emission spectra will always have a larger peak emission wavelength in comparison to the donor, allowing for relatively simple spectral separation of fluorescent signals. In terms of immunoassays, FRET has been demonstrated in three distinct formats using either two independent fluorescent labels or a label and a quencher. The first method involves the labelling of a single antibody with two chromophores. FRET signals are produced when a target biomarker binds to the antibody due to conformational changes in the antibody bringing the two chromophores into close proximity to one another, as shown in Fig. 3A/3B.^{29,39,40} The second method involves the independent labelling of two antibodies, which, following a given assay, brings the two labels in close proximity by the formation of the sandwich structure with a target biomarker, as shown in Fig. 3C.^{37,38} The third method involves the independent labelling of two antibodies, one with a fluorophore and the other with a quenching molecular label.

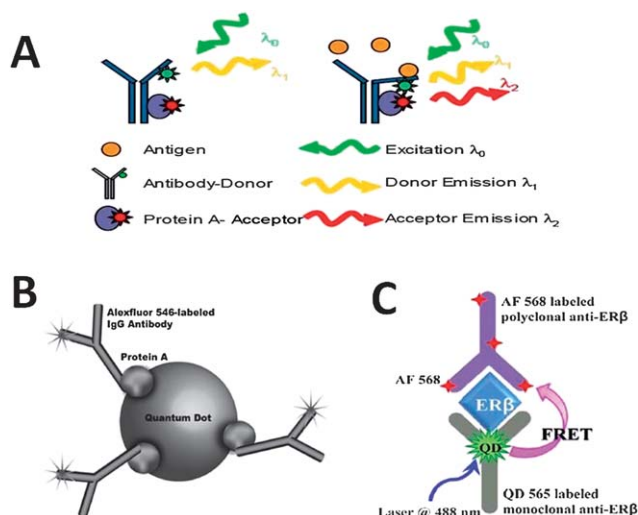


Fig. 3 All demonstrated FRET based immunosensor configurations. (A) A conceptual illustration of a dual organic fluorophore conformational change FRET based immunosensor by Pierce *et al.* 2004 (© 2004 IEEE).⁴⁰ (B) Self-assembled organic fluorophore/quantum dot conformational change FRET based immunosensor by Stringer *et al.* 2008 (© 2008 IEEE).²⁹ (C) Sandwich structure based organic fluorophore/quantum dot FRET based immunosensor by Park *et al.* 2004 (© 2004 KCS).³⁸

Following a given immunoassay, the label and quencher are in close proximity by the formation of the sandwich structure with a target biomarker, resulting in a drop in the fluorescence prior to binding.^{41,42} Both FRET techniques resulting from conformational changes and from interactions between a fluorophore and a gold nanoparticle quencher have been demonstrated in the detection of cardiac related biomarkers.

Using FRET based sensors, the spectral separation of the donor and acceptor emission does not necessitate the removal of the unbound, labelled antibodies. Potentially, FRET based bioassays can be completed within a single stage negating the washing phases typically required to remove unbound labelled antibodies, resulting in reduced total assay time and the operator demands. Disadvantages of FRET sensors are their low efficiency (5–15%) and the requirement of high efficiency optical filters and detectors. Additionally, these sensors operate at an increased cost per assay due to the use of the additional chromophores in comparison to single label strategies. FRET sensors have been demonstrated in the detection of cardiac related biomarkers cTnI^{29,39} and troponin T (cTnT).^{39,42} In the work by Grant *et al.*³⁹ both markers were detected with 75–100 nM detection limits using an optical fibre probe based immunosensor. Unlike other configurations, this sensor is placed into a test sample and may even hold potential as a biosensor for *in vivo* based cardiac marker measurements. In the work by Stringer *et al.*,²⁹ a conformational change based FRET sensor was developed to operate on the surface of QDs. In this biosensor the QDs act as the antibody immobilisation substrate in addition to a chromophore. This sensor was assembled such that excitation was achieved along the length of a liquid-core waveguide containing Alexa 546 labelled antibody conjugated quantum dots.

The detection of cTnI was achieved in human plasma samples with a 55 nM limit of detection, within a duration ranging 1–10 min. To date, this work seems to be the only example of a rapidly operating FRET based biosensor for use with human serum samples. In the work by Mayilo *et al.*,⁴² a microfluidic FRET based immunosensor was demonstrated for the detection of cTnT with a detection limit of 0.7 ng ml⁻¹. The immunosensor comprised the formation of the antibody-antigen sandwich structure, with one antibody labelled with a fluorophore and the other antibody labelled with a gold nanoparticle (AuNP). When in solution and prior to the introduction of the antigen, the fluorophore label is free to fluoresce upon excitation. Following the addition of the antigen, formation of the sandwich structure brings the fluorophore and AuNP to within the Förster distance, where the AuNP acts to quench the fluorophores emission fluorescence. The concentration of a given sample is therefore ascertained by the drop in fluorescence relative to a standard curve, with larger drops in fluorescence indicating a greater concentration of antigen. The use of AuNPs are advantageous as a quencher due to their operational efficiency being as high as 95%. An interesting FRET based device has been demonstrated in the work by Park *et al.*³⁸ using a microfluidic immunosensor to detect estrogen receptor β . The assay was demonstrated within an open air microfluidic device and despite not being utilised for the detection of cardiac biomarkers demonstrated a novel turbulence inducing, ‘alligator teeth’ shaped fluidic channel. The resulting configuration of the channel increases the mixing efficiency and, consequently, lowers the complete assay to within 3–5 min. Given the transferable nature of the immunoassay technology, this device and assay could readily be applied towards the detection of cardiac biomarkers.

A definite engineering challenge associated with the usage of chip based fluorescence intensity biosensors is in the miniaturisation of the excitation and detection apparatus required to perform a measurement, without significantly compromising detection sensitivities. In many instances the detection apparatus can be several orders of magnitude larger than the chip based sensors, despite the commercial availability of miniaturised photodetectors from most optoelectronic suppliers. Portable fluorescence detection instrumentation has been demonstrated in commercial POC devices (ESE Lateral Flow based immunoassay systems, QIAGEN and Cobas h232, Roche Diagnostics Ltd) and research grade fluorescence measurement systems for DNA bioanalysis,^{43,44} but has not seen integration in the majority of chip based immunosensing devices. The potential of current LoAC based fluorescence intensity cardiac biosensors for portable, POC applications is likely to be hindered until this challenge can be overcome and is anticipated to be the next logical phase of microfluidic based R&D.

2.1.2 Luminescence. Luminescence generating immunoassays are one of the earliest and most thoroughly researched biomarker detection strategies. As described previously, two primary mechanisms exist for the generation of the luminescent detection signal, either by the action of an enzymatic antibody label in the presence of a luminogenic substrate (chemiluminescence) or induced following an electron transfer reaction

of a luminescent compound immobilised near an electrode surface (Electrochemiluminescence, ECL).

Numerous test kits in the form of lateral flow devices are commercially available, the most well known being the home pregnancy test kit. Chemiluminometric immunoassays have a proven adequacy for use in POC applications and a cardiac marker detection system is commercially available (Cardiac-STATus, Nanogen). Measurements however remain largely qualitative, indicated by an unambiguous colour change above a pre-determined biomarker cut-off concentration. Commercial ECL based cardiac biomarker detection systems have also been demonstrated,^{50–52} with test kits to perform a more quantitative measurement, however, the equipment is bulky and not suitable for POC applications. What would be desirable is a more quantitative chemiluminescent and a more portable ECL based measurement system of cardiac biomarkers if the full POC potential of luminescent based devices is to be realised.

2.1.2i Chemiluminescence. More recently, chemiluminometric immunoassays have been examined for quantitative chip based measurements,^{45–49} including the detection of the cardiac specific biomarkers cTnI,⁴⁵ myoglobin⁴⁶ and CRP⁴⁷ at clinically significant concentrations. Demonstrated immunosensors predominantly operate by the formation of the antibody/antigen sandwich structure on the surface of the sensors, with the secondary antibody labelled with a reaction enzyme.^{45,46,48,49} The colour changing liquid substrate is then placed over the sensor area and the reaction enzyme begins to catalyse the mixture, leading to the detectable luminescent signal. An alternative sensor configuration has been demonstrated by Bhattacharyya *et al.*,⁴⁷ where the assay was conducted by the immobilisation of the target biomarker to the sensor surface, followed by the binding of an unlabelled antibody and subsequently a third enzyme labelled antibody. With this indirect ELISA method, no optical excitation is required, and with a sufficiently large concentration of enzyme and substrate, detection can be achieved by visual inspection. However, for quantification analysis, optical collection apparatus and a photodetector are necessary.

Most demonstrated chemiluminescent microfluidic based devices have their sensor element deposited on a solid substrate material,^{45–47} however, microparticle based configurations are also possible.^{48,49} The use of nanoparticles allows for a larger sensor surface area onto which antibodies may be immobilised and detection limits can therefore be improved. In the work by Yang *et al.*,⁴⁸ carbon nanotubes (CNT) were used as an immobilisation substrate and the resulting CNT-antibody complexes were immobilised onto a polycarbonate surface. Super paramagnetic particles (sPMP) have also been utilised as an antibody immobilisation substrate and captured in a magnetic trap placed within a microfluidic channel.⁴⁹ The magnetic beads have the advantage of being spatially addressable within the microfluidic channel over the region mounted with a photodetector and removable from the channel allowing for repeated use of the chip device, features not generally found with solid substrate based immunosensors. Nanoparticle based chemiluminescence immunosensors can achieve pg mL⁻¹ limits of detection, however, have yet to be demonstrated for cardiac biomarker specific detection.

In the work by Cho *et al.*,⁴⁵ a near complete chemiluminescent analysis system for the detection of cTnI is presented comprising of an absorbent material based flow through device as shown in Fig. 4. The device seems similar to the home pregnancy test kit, *i.e.* with reaction and control antibodies immobilised onto the surface of the absorbent material through which the test sample flows. The colour changing substrate is introduced by an absorbent pad placed orthogonally to the analysis section of the reaction pad and is retained in a secondary pad downstream. The device is capable of measuring the concentration of cTnI from serum samples as low as 27 pg mL⁻¹.

Commercial chemiluminescent based devices are currently utilised both clinically and for home users but remain largely qualitative in nature. The next generation devices are therefore expected to perform a more quantitative sample analysis. As with fluorescence intensity biosensors, the maturity of the chemiluminescence detection methodology has resulted in current research focusing more on challenges surrounding the integration of sensor elements into complete microfluidic and flow through platforms, to provide automation of the reaction process from sample introduction to detection.

2.1.2ii Electrochemiluminescence (ECL). ECL based immunosensing relies on activation of the luminescence from excited molecules, generally Ru(bpy)₃²⁺, which are generated from redox based electrochemical reactions in solutions containing a suitable reaction initiating substrate (*e.g.* tripropylamine). Unlike chemiluminescent sensors where enzymatic action begins upon direct contact of the enzyme and substrate, these sensors can be electronically activated by applying a reaction specific potential across the surface of an electrode.

Much of the current research has been predominantly focused on refinements of the biosensing technology and has seen novel immunosensing demonstrations using carbon nanotubes,⁵¹ various micro/nano particles^{51–53} and modified thin films.^{51,53,54} Currently, chip based ECL biosensing platforms have been less extensively examined by research groups, with very few demonstrated examples within the literature, and most focusing primarily on DNA based detection.^{55–57} Cardiac marker detection using ECL immunosensors has been demonstrated for myoglobin (1 nM detection limit)⁵² and CRP (10 ng mL⁻¹ detection limit).⁵³ Despite the relative maturity of ECL bio-analyte detection and the excellent work that is being conducted in this field, the general lack of demonstrated LoAC immunosensing devices requires further discussion. The excellent review of recent advances and future perspectives of ECL based detection by Bertoncello *et al.*⁵¹ provides a more in-depth treatment of the subject.

ECL has been widely utilised commercially for targeted cardiac biomarker detection for many years but has yet to be demonstrated in an integrated portable format. It is evident from the literature that numerous groups are developing immunosensor formats applicable for use within micro sized devices, enabled by the new generation of nanoparticle technology. The primary advantages of ECL immunosensing platforms are that they negate the requirement for an optical excitation source, provide a controlled means of initiating the luminescence producing reaction and is a highly sensitive means of bioanalyte detection. Currently demonstrated LoAC ECL platforms hint at

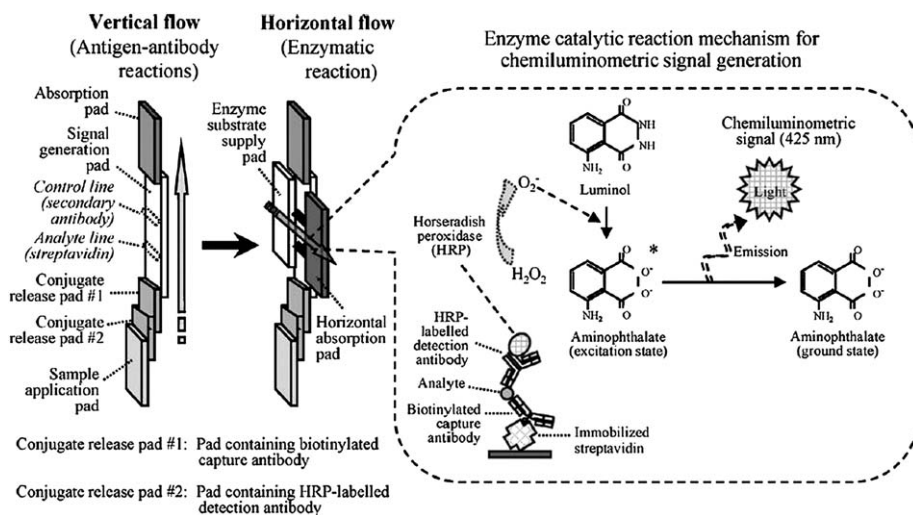


Fig. 4 A conceptual schematic of the cross flow chromatography with chemiluminometric signal generation from HRP catalysis as demonstrated in work by Cho *et al.* 2009.⁴⁵ The device was demonstrated for the detection of the cardiac specific biomarker troponin I, with detection possible over the concentration range of 0.1–100 ng mL⁻¹. Copyright © 2008 Elsevier.

the POC potential of this technology and it is anticipated that a portable and automated cardiac biomarker detection system will be demonstrated in the near future.

2.2 Surface plasmon based immunosensors

2.2.1 Surface plasmon resonance. Surface plasmon resonance (SPR) is a surface based optical technique which utilises the coupling of excitation light to a thin metallic surface, under exacting experimental conditions, which include the thickness of metal layer, the wavelength and angle of incidence of the excitation light.⁶⁵ In immunoassay based biosensors, the metallic surface is prepared with a layer of reaction specific immobilised antibodies. Binding of the target antigen leads to changes in the refractive index/thickness of the metallic sensor layer, resulting in a shift in the resonance curve of the reflected light that directly related to the concentration of bound biomarker. SPR biosensors are therefore advantageous as they do not require the labelling of the target antibody/antigen, reducing the cost and complexity of a given immunoassay. A potential disadvantage of SPR biosensors is that the sensor signal does not allow for discrimination of specific and non-specific binding of a target biomarker to the sensor area. This may become particularly problematic with respect to the analysis of biomarkers within challenging samples, such as blood, where non-specific binding is anticipated.⁶²

This limitation has been overcome by either the purification of the non-ideal sample prior to introduction over the SPR sensor area⁶¹ or by the modification of the biological assay performed on the sensor area.⁶⁴ The latter method involves the use of a secondary antibody which results in a secondary shift in the detected SPR signal after formation of the antibody/antigen sandwich structure.⁶⁴ This secondary shift provides validation that the target antigen has indeed been captured on the sensor due to the high specificity of the sandwich structure.

There are numerous different configurations by which SPR measurements can be performed with respect to immunoassays. The so-called 'Kretschmann configuration', shown in Fig. 5, is

the most prominent yielding $\mu\text{g mL}^{-1}$ down to ng mL^{-1} biomarkers detection sensitivities.^{58,60,62–64} In this configuration the biosensor consists of a glass prism coated with a metallic

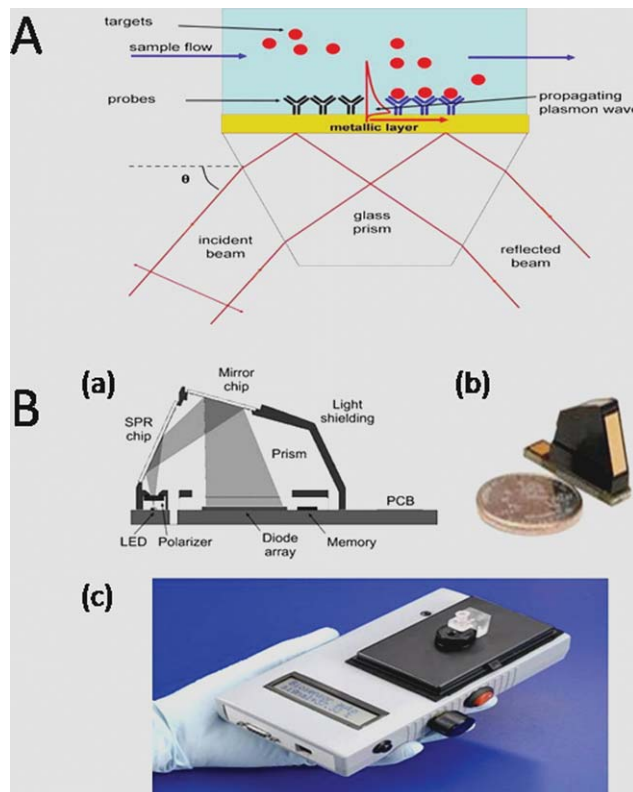


Fig. 5 (A) A diagram illustrating the general working principle of an SPR based immunosensor system, adapted from Duval *et al.*⁶⁰ © 2007 SPIE. (B) (a) A schematic of the Spreeta 2000 miniaturised SPR sensing device as demonstrated by Chinowsky *et al.*,⁶² © 2003 Elsevier. (b) A photograph of the Spreeta 2000 sensor pictured next to a coin (www.sensata.com), © 2010 Sensata Technologies Ltd. (c) A photograph of the handheld SPR device as demonstrated by Feltis *et al.*,⁶⁴ © 2007 Elsevier.

substrate (gold). The prism is used to increase the wave number of the excitation light and couple it to a thin metallic layer coated on the prism, under conditions of total and internal reflection. Under such conditions, an evanescent wave propagates through the metal layer, causing Surface Plasmon excitation on the outside of the metal, where the biosensor area is formed. Optical fibre based SPR sensors have also been demonstrated, with multimode fibres employed to direct excitation light and collect the reflected light from a purpose-made SPR sensor tip.⁵⁹

SPR detection is generally hindered by the required bulky detection apparatus, particularly for the Kretschmann configuration. Considerable engineering research has focused on the reduction in size and complexity of the sensor with various demonstrated solutions.^{62–64} For example, Chinowsky *et al.*⁶² present a disposable and miniaturised (15 mm × 7 mm × 3 mm) commercial SPR sensor (Spreeta 2000, Texas Instruments Inc.), shown in Fig. 5B. The device was demonstrated for the detection of IgG antibodies, yielding 80 pM limit of detection, which is comparable to performance of a standard ELISA immunoassay. A further example of a hand-held SPR immunosensor system has also been demonstrated in the work by Feltis *et al.*,⁶⁴ again utilising the Kretschmann configuration. The SPR sensor was sufficiently miniaturised by the use of a laser diode, simple optical lenses/mirrors and a photodiode array, all neatly packaged in a palm sized device, as shown in Fig. 5B. The sensor was demonstrated for the detection of Ricin with a limit of detection of 200 ng mL⁻¹.

SPR based immunosensing technology has been applied to the detection of cardiac specific biomarkers cTnI,^{59,69,70} myoglobin,⁵⁹ BNP,⁶¹ cTnT,⁶⁶ and CRP.^{67,68} Multimode optical fibre based SPR devices have been demonstrated for the detection of the cardiac biomarkers cTnI and myoglobin, with ng mL⁻¹ limits of detection.⁵⁹ The sensor has unique clinical potential for the real-time, *in vivo* POC monitoring of biomarkers through direct insertion into a vein. In the work by Kurita *et al.*⁶¹ a novel pre-conditioning microfluidic solution is presented by which thiol reaction products, resulting from an enzymatic immunoassay, are separated and immobilised onto an 'on-chip', gold film SPR sensor. Detection of the cardiac biomarker BNP was demonstrated with an impressive pg mL⁻¹ limit of detection. Further impressive detection limits have been demonstrated by Dutra *et al.* using a carboxymethyl-dextran-modified gold chip sensor substrate, where a detection limit of 10 pg mL⁻¹ was achieved when examining samples containing cTnT.⁶⁶

More currently, research groups are investigating the use of modified gold surfaces^{67,68} and gold nanoparticle based sensors^{69,70} for improved detection sensitivities. Such methodologies typically rely on the absorption characteristics of the examined system and so require simpler apparatus than the standard Kretschmann configuration. Sample concentrations are ascertained based on changes in the absorption intensity spectra at a characteristic UV-NIR wavelength, resulting from the formation of the gold-antibody-antigen complexes. The detection of cTnI has been demonstrated using both gold nanorods⁶⁹ and nanoprism structures⁷⁰ as the immobilisation substrate for the sensing antibodies. Both types of gold nanoparticles have allowed detection limits of 10 ng mL⁻¹. The cardiac biomarker CRP has been detected using an array style chip based format, with demonstrated detection limits of 100 pg mL⁻¹. As the device

was intended as a multiplexing device further details are found in the multiplexing section of this review.

SPR immunosensors have been demonstrated in a variety of formats, each of which offers excellent detection sensitivities across a variety of cardiac specific biomarkers. Considerable research has also enabled the miniaturisation of these detection platforms, dispensing with the need for bulky detection apparatus. Demonstrations of SPR immunosensor have achieved biomarker detection limits comparable to those obtained with direct fluorescence and chemiluminescent intensity measurements.^{61,66} However, they have yet to be demonstrated in the testing of challenging samples, such as blood, where potential hindrances to the biosensors capabilities, resulting from non-specific binding events, may significantly reduce the sensors sensitivity. These challenges must be addressed if these biosensors are to become useful for POC clinical diagnosis.

2.2.2 Metal enhanced fluorescence. Immunoassay labelling fluorophores, located within an appropriate distance from metallic nanoparticles or modified metallic films, can become strongly excited due to interactions of the emission fluorescence with freely mobile electrons in the metal, the so called 'lightning rod effect'. Observed metal surface interactions increase the radiative decay rate of a given fluorophore, translating into increased quantum yields and, correspondingly, an increase in detectable fluorescence intensity. These enhancements of the fluorescence intensity have been termed metal enhanced fluorescence (MEF) and, from theoretical calculations, could yield an increase in the number of detected photons per fluorophore by a factor of 10⁵.⁷⁵

To date, a limited amount of work has been conducted utilising the MEF effect in biosensors for the detection of the cardiac biomarker myoglobin. The main sensor format demonstrated consists of a chip substrate covered with a silver island film and coated with a layer of immobilisation antibodies.^{73,74} In the work by Aslan *et al.*,⁷⁴ the MEF sensor concept was investigated utilising a microwave cavity to kinetically accelerate the immunoassay reaction. The immunosensor, shown in Fig. 6, consisted of a silver island film substrate, onto which reaction antibodies were immobilised to allow for the formation of the sandwich structure, with an Alexa 647 labelled secondary antibody. The clinical cut-off concentration of myoglobin, 100 ng mL⁻¹, was investigated and the resulting fluorescence intensity could be clearly discriminated against background fluorescence. Additionally, the use of microwave energy allowed for the complete assay to be performed within 20 s, which is arguably the most rapid demonstration of an immunoassay biosensor. In the work by Matveeva *et al.*⁷³ a silver island film substrate was deposited on various biosensor substrates. Their work demonstrated that, through careful selection of the sensor substrate, the fluorescence emission on a mirrored layer can increase approximately by a factor of 10 compared to silver islands placed on a glass substrate.

Fluorescence based enhancements have also been widely documented in a wide variety of bioassays using gold nanoparticles. In work by Hong *et al.* the fluorescence enhancement properties of AuNP, resulting from the surface plasmonic fields, were utilised to increase the detection capabilities of an optical fibre based immunosensor.⁷⁶ The sensor comprised of

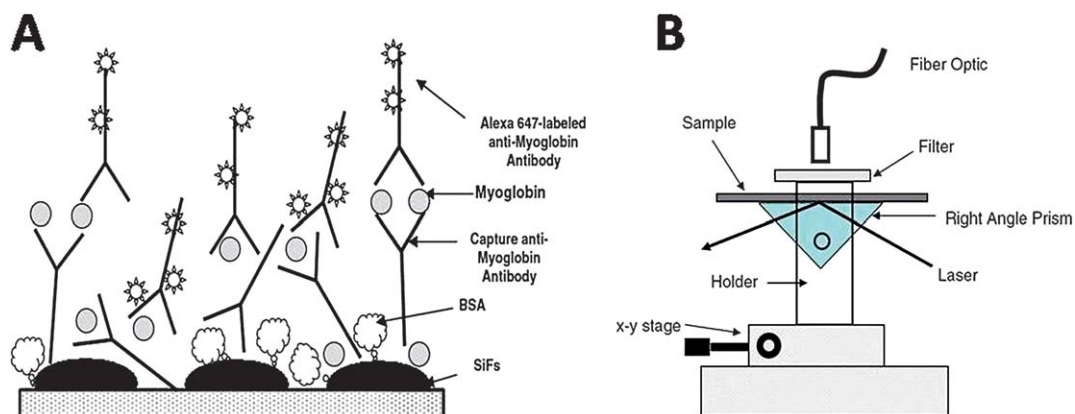


Fig. 6 Diagrams illustrating the immunosensor and detection apparatus as devised by Aslan *et al.* 2006.⁷⁴ (A) A schematic of the MEF silver island film immunosensor following antigen and secondary antibody capture. (B) A schematic of the detection apparatus used to image the sensor area. © 2006 Springer Scientific.

antibodies immobilised onto the sensor surface and detection was achieved by the formation of the sandwich structure, with the fluorophore labelled secondary antibody, followed by an incubation phase with modified AuNPs. This sensor was utilised for the detection of the cardiac biomarkers cTnI (0.7 ng ml⁻¹ LOD), myoglobin (70 ng ml⁻¹ LOD), BNP (0.1 ng ml⁻¹ LOD) and CRP (700 ng ml⁻¹ LOD). The complete device was packaged into a complete, multiplexed microfluidic device format, comprising of an impressive array of various microfluidic valves, pumps, channels and a turbulence inducing structure, neatly packaged into a prototype device. The impressive detection and fluidic manipulation capabilities of this device demonstrate considerable potential for POC cardiac biomarker applications.

Current work shows considerable promise for the future of the MEF technique for immunosensor applications both for sensors upon a metallic substrate and metallic nanoparticles. Work by Hong *et al.* has demonstrated such sensors can be used alongside microfluidic based platforms in a complete device format. Given the majority of demonstrated micro sized devices utilises a fluorescence intensity based measurement to determine cardiac marker concentration, then MEF sensors would only serve to further enhance such devices.

2.3 Raman spectroscopy based immunosensors

2.3.1 Surface enhanced Raman spectroscopy. In Raman spectroscopy unique 'spectral fingerprints' are produced following the inelastic scattering of photons incident on an examined molecule. This inelastic scattering occurs when incident photons lose their energy to the quantum vibrational modes in the target molecule, resulting in the unique 'spectral fingerprint', consisting of Stokes-shifted peaks. This effect allows for Raman spectroscopy to be a label free technique as the resulting vibrational fingerprint is based solely on the target molecule. surface enhanced Raman spectroscopy (SERS) is a surfaced based optical technique by which a Raman signal is enhanced following immobilisation of the target molecule onto a rough metallic surface, such as silver or gold. The precise mechanism by which this enhancement occurs is still currently unknown.^{87,88}

The enhancement of the Raman signal has been reported to be as high as a factor of 10¹⁴–10¹⁵, which is of an adequate sensitivity to facilitate single molecule detection.⁸⁸ This enhancement however decreases exponentially with the distance from the metallic surface.

SERS immunosensors have demonstrated detection limits of pg mL⁻¹ concentrations,^{77,84} which is several orders of magnitude lower than many fluorescence intensity based immunosensors. Demonstrated immunosensor formats largely consist of the antibody/antigen sandwich structure formation with primary antibodies immobilised on a sensor substrate and the secondary antibodies labelled with a metallic Raman reporter molecule.^{77–79,83,84} This process mirrors the one of the fluorescent labels, however, SERS labels produce virtually no background, thus improving the signal to noise ratio and in turn the sensitivity of the biosensor. Moreover, SERS nanoparticle labels do not photobleach and produce narrow and tuneable emission spectra making them suitable for multiplexed immunoassays.⁷⁹ Limitations of the use of metallic nanoparticles primarily relate to antibody binding, particularly limitations due to nanoparticle biocompatibility and the non-uniform binding surfaces as 'rough' metallic surfaces are desirable for increased Raman signal. This issue has been addressed in the work of Mulvaney *et al.*⁷⁹ where glass coated Au/Ag nanoparticles have been created with the aim of producing more robust and biocompatible reporters with a more consistent and uniform surface. The use of a glass surface is advantageous as it allows for the exploitation of the optical and electrical properties of the reporter, while being an established biocompatible substrate, suitable towards immunoassays applications. However, glass suffers from auto-fluorescence and can therefore hinder optically based detection assays.

Modifications to the metallic nanoparticle Raman labels have been reported with the aim of further increasing SERS sensitivities and developing multiplexed immunosensor formats.^{78,83,84} These modifications include silver staining enhancement, which has been demonstrated to yield a 10–100 fold improvement of a comparative SERS signal.⁸³ Further increases in sensitivity can be achieved by the labelling of metallic nanoparticles with a unique reporter molecule.^{78,84} This use of different reporter

molecules can allow for multiplexed reactions and shall be discussed later in this review.

Despite all the current research in the field of SERS immunosensors, a minimal amount of work has been conducted specifically on the detection of cardiac related biomarkers. This may be due to research groups opting to utilise SERS based detection, an adaptation of the SERS technique with increased detection sensitivities, as explained in the next section. Work by Grubisha *et al.*⁸⁴ demonstrates that the SERS technique may be used to examine serum samples with no requirement for sample pre-conditioning and so this technique may prove useful for POC based biosensor applications where serum samples are predominantly tested.

2.3.2 Surface enhanced resonance Raman spectroscopy. More recently, the SERS technique has been developed to make use of enhancements resulting from the resonant excitation of molecules. This technique, known as surface enhanced resonance Raman spectroscopy (SERRS), typically involves the detection of the Raman signal resulting from a chromophore/chromogen labelled metallic nanoparticle, excited at a frequency centred on the maximum of absorption of the label.^{80–82,90,91} The net effect is a molecular resonance contribution, added to the surface enhancement, leading to increased detection sensitivities compared to SERS. There is much choice over the selection of Raman probe label with demonstrations ranging from standard chromophores^{86,90–92} to enzymatically reacting chromogens.^{80–82}

Demonstrations of immunosensors largely consist of enzymatic based immunoassays operating by formation of the sandwich structure with an enzyme label, an example of which is shown in Fig. 7.^{80–82} Solutions containing chromagens and metallic nanoparticles are introduced over the sensor area. Following the action of the enzyme label, the chromagen becomes optically active and absorbs onto the surface of the nanoparticle. The labelled Raman reporter is then detected.

SERRS immunoassays have been demonstrated using gold^{80,81} and silver⁸² nanoparticle Raman reporters, with demonstrated target antigen detection of the order of ng mL^{-1} down to pg mL^{-1} concentrations. Currently, there is limited literature available of the targeted detection of cardiac related biomarkers, but existing demonstrations show very much promise. In work by Bizzarri *et al.*,⁸⁹ the cardiac marker myoglobin was detected on silver colloidal nanoparticles with a claimed single molecule detection capability. CRP was detected in the work by Campbell *et al.* at concentrations as low as 0.3 ng mL^{-1} , where clinically relevant concentrations are of the order of $\mu\text{g mL}^{-1}$.⁸⁰ Both examples show impressive detection sensitivities when applied to cardiac marker concentration measurements, demonstrating thereby the potential of this technique for POC applications.

Limited literature is also available about the demonstration of chromophore Raman reporter immunosensors,⁸⁶ possibly due to the difficulties in devising a suitable immunoassay format. Chromophore Raman reporter SERRS is made possible due to the metallic surface quenching the fluorescence from the chromophore.⁹⁰ Maintaining this requirement and integrating the Raman reporter into an immunoassay format becomes a non-trivial problem. This challenge was addressed in work by Han *et al.*⁸⁶ where an immunoassay format was created which used silver nanoparticles to act as an amplifier for the resonance Raman signal of FITC labelled antibodies. The assay comprised of antigens immobilised on a substrate, which were incubated with FITC labelled antibodies. A solution of colloidal silver nanoparticles was then incubated over the bound antibodies leading to silver staining. On completion of the silver staining the SERRS spectrum of the FITC was retrieved, yielding 0.2 ng mL^{-1} limits of detection for human IgG. This assay format has yet to be demonstrated for the detection of cardiac biomarkers and may currently be limited by the requirement to immobilise the target antigen to a substrate, which may be impractical when complex samples, such as blood, are required for examination.

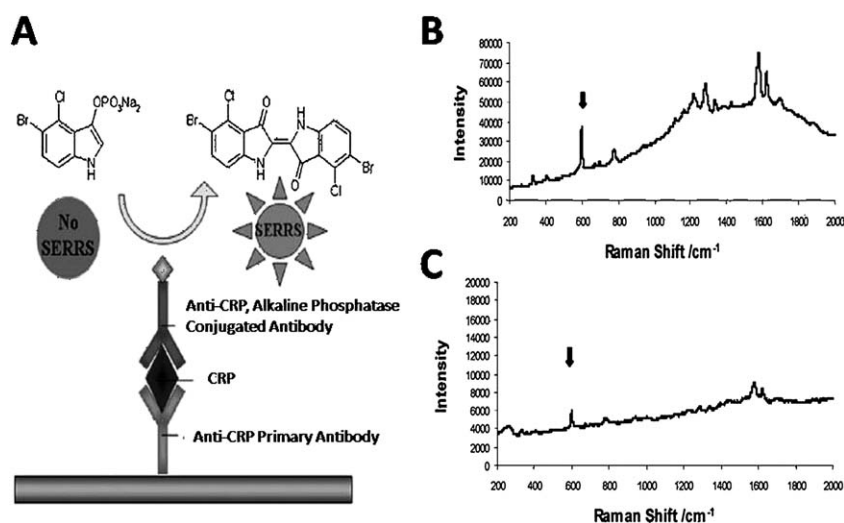


Fig. 7 Diagrams illustrating the SERRS based immunosensor and the resulting SERRS spectra for the capture of CRP antigens, as demonstrated by Campbell *et al.* 2008.⁸⁰ (A) A concept diagram of SERRS immunosensor illustrating the conversion of BCIP into a SERRS-active species upon exposure to alkaline phosphatase. SERRS spectra obtained for the detection of (B) 100 ng mL^{-1} and (C) 2.5 ng mL^{-1} of CRP. © 2008 The Royal Society of Chemistry.

3 Electrochemical based immunosensors

Electrochemical immunosensors have received considerable attention by research groups over the past decades, with a wealth of publications and demonstrations of novel and unique detection platforms. Consequently, this review shall give the reader only a brief overview of demonstrated detection methodologies and focus more on examples of cardiac marker related electrochemical sensors. In broad terms, electrochemical based immunosensors operate by the detection of an electrical signal resulting from specific immunoreactions. There is much versatility in the selection of detected signal by which analyte concentrations can be determined, with demonstrations including amperometric,^{93,97,99,101,102} impedimetric^{94,95,103,105,106} and potentiometric^{93,95,100,103–105,107–111} based detection signals. Electrochemical immunosensors can be readily fabricated from a range different conducting substrates using standard chip based manufacturing methodologies, making them highly desirable from a manufacturing perspective.

3.1 Amperometric

Amperometric immunosensors operate by the measurement of the current generated at a working electrode due to a redox reaction on the surface of the electrode following an immunoreaction. These sensors are generally enzymatic based and operate by the formation of the standard antibody/antigen sandwich structure created either upon the electrode surface or upon the surface of a reaction membrane, with the secondary antibody labelled with a redox initiating molecule/enzyme. The current generated due to redox of the working electrode is therefore proportional to the number of bound labelled secondary antibodies and allows for quantification of bound target antigen concentrations. During the reaction the working electrode is maintained at a specific potential. Changes in the current are measured relative to a reference electrode, typically an Ag/AgCl electrode, which is kept at thermodynamic equilibrium.

In the work by Siegmann-Thoss *et al.*⁹⁷ an amperometric immunosensor was created, utilising a Clark-type oxygen electrode covered by a nylon membrane functionalised with anti Heart-Fatty Acid Binding Protein (H-FABP) antibodies. H-FABP is a cardiac risk marker implicated in various forms of MI. The sensor consumes oxygen in a reaction with glucose oxidase antibody labels, which diffuse through the membrane and are reduced at the surface of the Clark-type working electrode. The immunosensor allowed for H-FABP detection over the range of 5–100 ng mL⁻¹ and tests performed on spiked serum samples yielded an average recovery of 93 ± 5%. This detection capability is very impressive with detection limits comparable to modern optical based immunosensors. The more recent work by O'Regan *et al.*^{101,102} details the use of a disposable type amperometric immunosensor comprising of screen-printed electrodes made of carbon. The sensor operates by performing a one-step sandwich immunoassay on the sensor surface, with the secondary antibody labelled with alkaline phosphatase (AP). Current signals are generated by AP oxidising *p*-aminophenyl phosphate to *p*-aminophenol. Using clinical blood samples, the immunosensor was demonstrated for the detection of the cardiac specific biomarkers



Fig. 8 Picture of the i-STAT POC system (Abbott Point of Care, U.S.A). <http://www.abbottpointofcare.com/ISTAT>. © 1997–2010 Abbott Laboratories.

myoglobin with a detection range of 80 to 925 ng mL⁻¹¹⁰² and H-FABP with a detection range of 4 to 250 ng mL⁻¹.¹⁰¹

Amperometric immunosensor research has matured considerably in recent years, to the point where commercial POC immunosensing systems are currently available for targeted cardiac biomarker detection. The i-STAT (Abbott Point of Care, U.S.A), shown in Fig. 8, is a commercially available amperometric based immunosensing device capable of measuring the concentrations of cTnI, CK-MB and BNP cardiac markers directly from 16 µL samples of whole blood, by means of a cartridge system in which all the biological reactions occur.¹⁴⁷ The device is capable of detecting cTnI over a range of 0–50 ng mL⁻¹ and has already gained much acceptance for medical usage as a diagnostics tool for MI.

3.2 Potentiometric

Potentiometric sensors operate by examining the potential difference either between two reference electrodes separated by a permselective membrane or by a working and reference electrode when no current flows between the electrodes.¹⁰⁷ The potentiometric signal is typically generated by changes in the pH, redox or ionic state, resulting from the specific biological interaction at the surface of the sensor. Potentiometric biosensors can be broadly categorised as either ion-selective electrodes (ISE),¹⁰⁰ field effect transistor (FET) type sensors^{104,109,110} or as light addressable potentiometric sensors (LAPS).^{103,108,111} FET sensors are an adaption of the ISE format, in a sense that the electrode is replaced with the FET transducer. Such sensors appear to be the preferred format for immunosensing purposes.

A common approach to potentiometric based immunosensors is to perform the standard sandwich structure forming immunoassay on a reaction substrate, using an enzyme labelled secondary antibody. The potentiometric signal results from the adsorption of catalysed substrate molecules onto the desired biosensor surface. Such a format has been demonstrated as an ISE potentiometric immunosensor in the work by Purvis *et al.*¹⁰⁰

using a polypyrrole coated gold electrode sensor. Following the antibody/antigen sandwich structure formation, a horseradish peroxidase (HRP) enzyme label converts *o*-phenylenediamine into 2,3-diaminophenazine in the presence of H_2O_2 . The reaction leads to redox, pH and ionic events which change the physical and electrochemical properties of the polypyrrole layer, resulting in a shift in the potential of the sensor. This shift directly relates to the concentration of bound antigens from the immunoassay reaction. The immunosensor was demonstrated for the detection of a variety of targets analytes, including the cardiac specific biomarker cTnI, yielding an impressive detection sensitivity of 10 pg mL^{-1} .

FET based potentiometric immunosensors have been demonstrated in a variety of novel formats, each resulting from unique immunosensing reaction configurations for signal generation. In work by Besselink *et al.*,¹⁰⁹ an ion sensitive FET (ISFET) immunosensor was demonstrated, comprising of human serum albumin (HAS) immobilised onto a monolayer of latex beads, which in turn were functionalised onto an ISFET surface. This immunosensor operates utilising an ion step response, where the potentiometric signal is generated by the release or uptake of protons from proteins bound upon the

surface of the sensor. The device is capable of producing the ion step response following the binding of HSA antibodies onto the sensor, surface bound HSA. The device served more as a demonstration of the technique and no results were presented of the detection limits of the sensor. An alternative FET based immunosensor is demonstrated in the work by Kamahori *et al.*¹¹⁰ consisting of a gold electrode and a FET structure. The sensor was made to operate within a microplate well containing immobilised antibodies for Interleukin 1β and the standard antibody/antigen sandwich structure is formed following capture of the target antigen as shown in Fig. 9A. The secondary antibody is labelled with AChE and following enzymatic catalysis of acetylthiocholine, generates a thiol compound on the surface of the gold electrode. The FET structure transduces this chemical event on the gold electrode into a potentiometric signal allowing thereby the detection of Interleukin 1β over a range of $1\text{--}250 \text{ pg mL}^{-1}$. The high sensitivity of FET based potentiometric immunosensors make them desirable towards POC application for the detection of cardiac specific biomarkers and other biological analytes, however, such demonstrations have yet to be realised.

There is a limited number of demonstrated LAP immunosensors formats found in the literature, despite the prominent

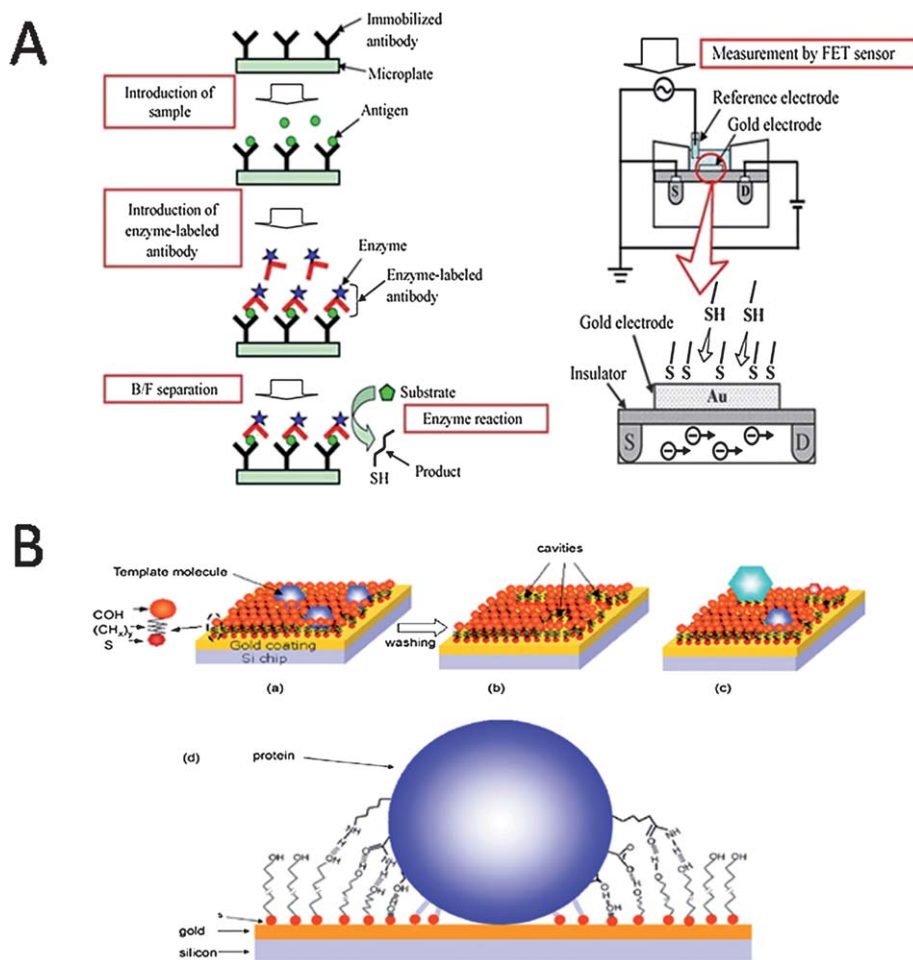


Fig. 9 (A) Modified diagram presented in the work by Kamahori *et al.* 2007 describing the principle of an enzyme immunoassay using an extended-gate FET biosensor.¹¹⁰ © 2007 Elsevier. (B) A diagram describing the fabrication and proposed working mechanism of the protein imprinted immunosensor as demonstrated by Wang *et al.* 2008.⁹⁶ © 2008 Elsevier.

usage of LAPs for alternative sensor formats. LAP sensors generally consist of a silicon sensor substrate that is activated by a modulated LED light source, producing an alternating photocurrent with a characteristic current and voltage profile. The LAP sensor acts as an electrode which typically measures the voltage per time differentials resulting from a change in the pH of a reaction medium, effectively operating as a pH electrode. In the work by Gehring *et al.*,¹¹¹ a LAP immunosensor was demonstrated for the detection of *E. coli* cells. The sensor involves the formation of the sandwich antibody structure on a nitrocellulose membrane, with the secondary antibody labelled with urease conjugate. Urease is used to catalyse urea to form ammonia, which alters the pH of the reaction medium resulting in a potential difference. The LAP immunosensor was capable of a limit of detection of 710 cells mL⁻¹ of *E. coli*. In the work demonstrated by Ercole *et al.*,¹⁰⁸ this LAP sensor arrangement was refined and *E. coli* detection limits were improved to 10 cells mL⁻¹. To-date the LAP immunosensor format has yet to be demonstrated for the detection of cardiac specific biomarkers. As with amperometric immunosensors, LAP sensors are a mature field of investigation and have consequently led to the creation of a commercial device called the Threshold Immunoassay System® (Molecular Devices, US). This device is currently in use for a host of different immunoassay based applications, including use in the evaluation of drug clearance and various immune responses from clinical samples.

A novel form of potentiometric immunosensor has recently been demonstrated through the work of Wang *et al.*⁹⁶ who print at the molecular level a thiol self assembled monolayer (SAM) onto a gold coated silicon substrate. The sensor, shown in Fig. 9B, is formed such that a solution containing a low concentration of template protein molecules and thiol are left to form the SAM on the gold sensor surface. The template protein molecules are only held to the surface by hydrophobic interactions/electrostatic forces and so can be removed after SAM formation, leaving free cavities into which the target globular proteins can be captured. The capture of proteins in the cavities increases the potential of the sensor relative to an Ag/AgCl reference electrode, with the resulting potential being proportional to the concentration of captured analytes. The sensor has been demonstrated for the capture of haemoglobin and of the cardiac biomarker myoglobin at detection limits of 1–100 µg mL⁻¹. Although the construction and operation of the sensor are simple, the detection sensitivity of the sensor fails to match the performance of alternative potentiometric based immunosensors by several orders of magnitude. Further development would be necessary if such a sensor were to become useful for practical POC applications.

3.3 Impedimetric

Impedimetric biosensors operate by a change in impedance, resulting from changes in resistance or capacitance at the sensor surface following a given immunoreaction. In a typical sensor, reaction antibodies are immobilised onto the surface of a sensing electrode and antigen binding results in the formation of an insulating layer, which in turn increases the impedance of the sensor. The increase in impedance is thus directly related to the concentration of bound antigens allowing for quantification of

the sample concentration. Impedance measurements are made using a standard three electrode system, with applied AC voltages of 10–100 mV and comprising of a working electrode, a counter electrode (Pt) and a reference electrode (Ag/AgCl^{94,95} or saturated calomel electrode¹⁰⁶). The biosensor operates as part of a Wheatstone bridge circuit and various AC voltage signals, with amplitudes over 1–100 mV, are applied between the measurement electrodes. The working electrode generally comprises of a gold electrode functionalised with a self assembled monolayer, due to their well studied surface chemistry, which readily allows for antibody binding.^{94,95,106} An immediate advantage of impedimetric biosensors is their ability to operate without the need of an antibody label, however, such sensors have been criticised due to potential false positives resulting from undesired electrolytes in a sample.¹⁰³

More recently, impedimetric immunosensors have been developed for the detection of cardiac specific markers, with demonstrations for myoglobin^{94,95} and CRP detection.¹⁰⁶ In the work by Tweedie *et al.*,⁹⁵ results were presented of a microfluidic based impedimetric immunosensor shown in Fig. 10, capable of detecting the cardiac biomarker myoglobin. A completely packaged microfluidic chip based system is demonstrated for myoglobin detection at the clinical upper limit of detection (100 ng mL⁻¹). The presented device shows much promise for future designs and elegantly demonstrates the simplicity by which impedimetric immunosensors can operate and be integrated into microfluidic designs. Myoglobin detection has also been demonstrated on a mixed self-assembled monolayer immunosensor in the work by Billah *et al.*⁹⁴ The mixed monolayer consists of alkanethiols and phospholipids which are believed to provide greater flexibility in the directional orientation of the immobilised sensing antibodies. The sensor was capable of myoglobin detection over concentrations of 10⁻¹²–10⁻⁶ M, from

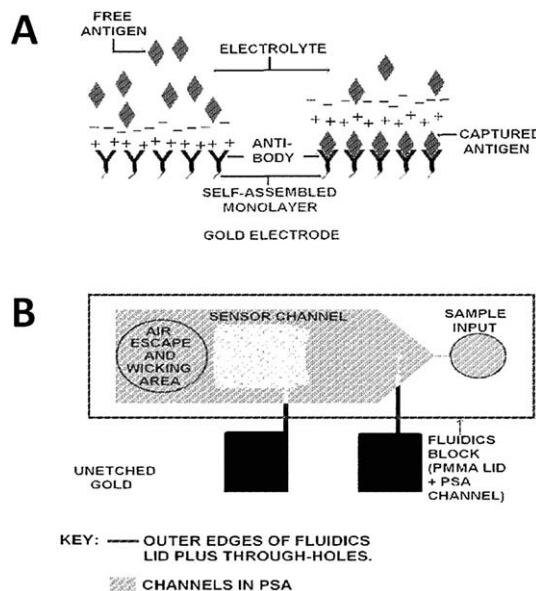


Fig. 10 Diagrams illustrating (A) the immunoassay and (B) the microfluidic chip device used to perform impedimetric measurements of myoglobin from whole blood, as demonstrated by Tweedie *et al.* 2006.⁹⁵ © 2006 IEEE.

samples of 10% and 100% serum, where clinical concentrations are approximately 10^{-10} M or 15 ng mL^{-1} . Such detection sensitivities are highly comparable to the most sensitive, alternative immunosensor formats and demonstrate the capacity of impedimetric immunosensors to use clinical test samples, hinting at their POC potential. In work by Chen *et al.*,¹⁰⁶ an immunosensor comprising of a three dimensionally ordered macroporous (3DOM) gold film was developed for the detection of the cardiac marker CRP. The 3DOM gold surface possesses a larger active surface area than SAMs, leading to increased conductivity and a larger sensor antibody immobilisation surface. The sensor allowed for the detection of CRP in the range of $0.1\text{--}20 \text{ ng mL}^{-1}$ in serum samples and measurements were in agreement to clinically obtained results, from identical test samples. The sensitivity of the 3DOM based immunosensor is less than that of the mixed monolayer sensor, which implies that further refinements may be required to maximise the full potential of this type of sensor type. However, both sensors have not been tested using identical antibodies for the detection of the same target biomarker, where binding efficiencies and, consequently, the detection sensitivity may be altered.

Impedimetric immunosensors show much promise for the future POC applications given their chip sized dimensions. They can readily be utilised directly with clinical samples and have detection sensitivities beyond the clinical requirements for cardiac specific biomarker detection. The work by Tweedie *et al.* has shown that such sensors can be readily packed into a microfluidic, chip based design, further enhancing the potential of such sensors for POC applications and it is anticipated that a commercial device, much like the i-STAT, may become a reality in the forthcoming years.

4 Paramagnetic particle based immunosensors

Paramagnetic particles (PMPs) typically consist of an iron oxide core coated with a polymer layer. Such particles are readily available in micro and nano sizes from a host of commercial distributors. The iron oxide core can be coated with polymers with a high biocompatibility, allowing PMPs to be developed towards biosensing applications. Consequently, PMPs have been demonstrated in applications such as DNA hybridisation assays, DNA and biochemical separation, MRI contrast agents¹¹⁷ and within immunoassay formats.^{112–117} The advantages of PMPs for biosensing applications stems from their unique magnetic properties which allow them to serve as both a reaction label^{112–114,116,117} and as a transportable sensor surface.¹¹⁵ In this sense PMPs share many of the same benefits as QDs, however have the further advantage that the motion of PMPs can be influenced by an imposed magnetic field, allowing for capture and transportation when utilising their particle properties for sensing platforms. More detailed information relating to the various magnetic based biosensor configurations can be found in the highly informative review conducted by Graham *et al.*¹¹⁸

The majority of demonstrated PMP based magneto-immunosensors involves the formation of the antibody/antigen sandwich structure on the surface of a solid substrate, with the PMP acting as the reaction label.^{112–114,116,117} Such sensors generally operate in an open chamber chip sized format, with reaction surfaces formed from a variety of biocompatible substrates.

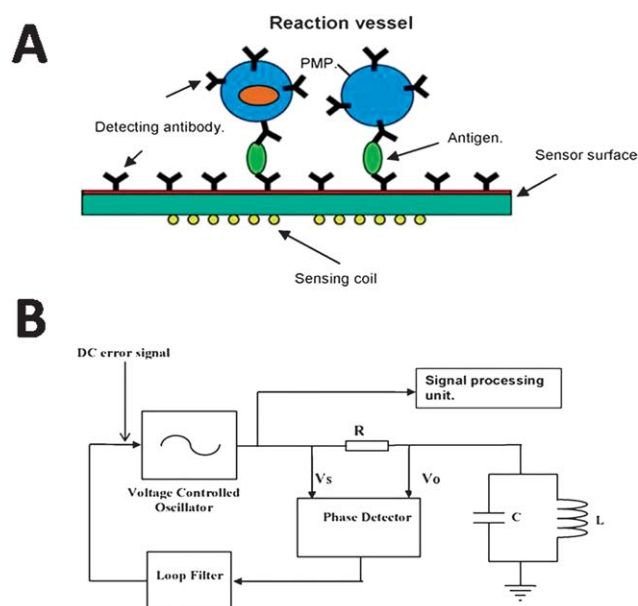


Fig. 11 Diagrams illustrating (A) the immunosensor and (B) the phase lock loop detection circuit for the PMP based immunosensor as demonstrated by Eveness *et al.*¹¹⁷ © 2009 Elsevier.

Reagents are delivered manually onto the sensor surface by pipetting, with demonstrated designs having less emphasis upon incorporation of fully automated flow cell/microfluidic reagent delivery. The magnetic properties of PMPs can also be used as a means of reagent mixing, transportation to the surface of the sensor and for removal of any unbound PMP labelled antibodies.^{112,113,117} The sensor surface is orientated directly above a sensing coil, which forms part of LC circuit driven by a voltage controlled oscillator at a precise resonant frequency. Following an assay, PMPs immobilised on the sensor surface lead to an increase in the sensor coil inductance, resulting in a frequency shift of the LC circuit. The increase of inductance only arises when the PMPs are in close proximity to the sensing coil. This occurs following the formation of the sandwich structure on the sensing surface. Such sensors tend therefore to suffer less from background signals and can allow for the reaction to be performed in a single step.^{112,113,117} The shift of the frequency response is proportional to the number of bound PMP labels, which in turn is directly related to the concentration of captured target antigen. As the total mass of bound PMPs affects the frequency response, the use of larger iron oxide particles can increase the sensitivity of such sensors. Work characterising a variety of commercially available PMPs of various sizes can be found in work by Eveness *et al.* as shown in Fig. 11.¹¹⁷ Magneto-immunosensors have been demonstrated in the detection of the cardiac specific markers cTnI,¹¹² CKMB and CRP,¹¹³ achieving clinically relevant detection sensitivities for each marker of the order of ng mL^{-1} . Such sensors are therefore good contenders for POC applications but require further developments with respect to fluidic delivery and usage with real clinical samples. There may be, for example, engineering challenges related to the use of such sensors with blood samples due to blood haemoglobin containing iron and therefore being susceptible to the influence of a magnetic field.

An adaption of the magneto-immunoassay is to perform detection using a superconducting quantum interference device (SQUID).¹¹⁶ In this detection method, the previous stages leading to sandwich structure formation are performed and subsequently an external magnetic field of 0.1 T is applied perpendicularly to the sensor to align the magnetic moment of each immobilised iron oxide label. The sensor is then placed into the SQUID and the remanence field is measured, which directly relates to the concentration of captured target antigen. SQUID detection systems have been demonstrated to be highly sensitive. In the work by Enpuku *et al.*,¹¹⁶ IgG was detected at concentrations as low as 2 attomoles. This highly impressive demonstration is arguably the most sensitive immunosensor across all demonstrated sensor types. This type of sensor might not be suitable to POC applications due to the bulk of the detection apparatus and the requirement for the SQUID to be maintained at ~ 77 K.¹¹⁴

5 Mass-loading methodologies

In mass-loading immunosensors an antibody is immobilised on a sensor surface. Following an immuno-reaction binding event, the surface of the sensor is physically displaced from an equilibrium position, resulting from the additional mass of the captured antigen or protein complex. This ultimately results in a detectable signal (frequency shift, laser beam displacement) that directly relates to the concentration of the target antigen. Two distinct technologies have been demonstrated, microcantilevers^{119–126} and acoustic wave sensors,^{127–130,132–146} both of which can be utilised in label free detection methods.

5.1 Microcantilever based immunosensors

Microcantilever (MC) based immunosensor technology has primarily resulted from advancements in fabrication techniques, allowing for the production of high quality, nanometre thick lever type structures. MC sensors are sensitive to mass loading

upon their surface resulting in an observable displacement from an equilibrium position or to changes in its vibrational frequency. MC sensors can be fabricated from various semiconductor and metallic substrates; their structure is akin to that of a swimming 'diving board' of nano to micrometre dimensions.^{119–124,126} Although less common, alternative structures are possible, such as double legged¹²⁶ and triangular^{125,126} shaped cantilevers.

Two distinct variants of MC have been demonstrated: those which integrate a piezoelectric material near to the upper surface of a lever as shown in Fig. 12A^{120,123} and those comprising of basic silicon/metal structures as shown in Fig. 12B.^{121,122,125} The latter, simpler MC structures are examined in terms of the physical deflection of the lever due to mass loading, where the deflection is realised based on spatial detection of a reflected laser beam, focused on the apex of the MC. This methodology is highly sensitive and is capable of detecting sub-nanometre MC deflections; however, the laser beam can cause undesirable thermal effects, limiting the potential sensitivity and can lead to erroneous readings.^{123,126} Further issues can arise related to the alignment of the laser system both onto the MC and to the photodetector. This can be overcome by the use of high precision optical apparatus but reduces the desirability of the sensor for POC applications due to the increased associated complexity and costs.¹²⁰ Piezoelectric MCs rely on a measurable resonant frequency change of an input AC sine wave due to a strain in the piezoelectric material, resulting from a stress change of the cantilever due to mass loading upon its surface.^{120,123,126} Such sensors are advantageous as they require less stringency with respect to the detection apparatus, much of which can be integrated on chip. Piezoelectric MCs have a lower detection sensitivity compared to laser deflection detection and a more complex fabrication process although they do not suffer from the thermal effects induced by the laser system.

When used as immunosensors, MCs are prepared by suitable modification of the cantilevers surface to allow for the attachment of reaction capture antibodies.^{121–125} Immunosensors have been demonstrated using both standard and piezoelectric based MC configurations for the detection of a host of different target biomarkers, including the cardiac specific biomarkers myoglobin,^{120,122} CKMB¹²² and CRP.¹²³ One of the most impressive demonstrations was presented in the work by Wu *et al.*,¹²⁵ where a laser deflection based sensor achieved 0.2 ng mL^{-1} detection of PSA antigen from samples containing human serum and plasminogen. This limit of detection is comparable to that achieved by a standard ELISA assay and is one of the only demonstrations of a MC immunosensor operating with standard clinical samples, highlighting the potential of this sensor type for POC applications.

MC based immunosensors represent a novel type of immunosensor, with a certain elegance resulting from their simplicity of usage. Compared to traditional immunoassay formats such as ELISA, MC immunosensors require less operator demands to perform the assay, whilst still achieving comparable detection sensitivities.¹²⁵ In addition, MC immunosensors have been tested using samples similar to those found within a clinical setting and for the detection of cardiac biomarkers, demonstrating the applicability of such sensors for POC purposes. An issue that has yet not been addressed is the packaging of the MC based sensor

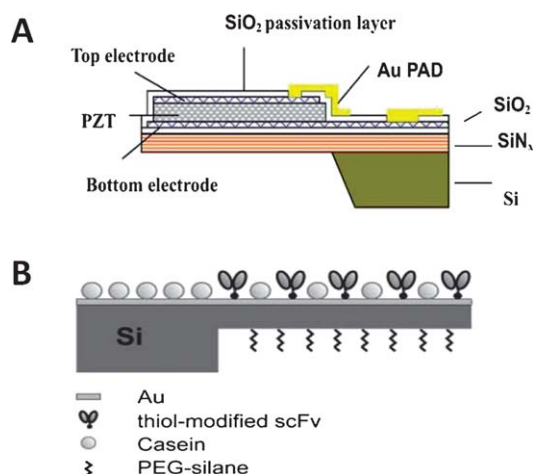


Fig. 12 Diagram illustrating the two demonstrated variants of microcantilever based immunosensors. (A) piezoelectric based microcantilever as demonstrated by Lee *et al.*,¹²³ © 2004 Elsevier. (B) Silicon/gold based microcantilever sensor as demonstrated by Backmann *et al.*,¹²¹ © 2005 The National Academy of Sciences of the USA.

into a portable device especially for laser deflection based mechanisms. Piezoelectric configurations, due to the reduced complexity and stringency of the detection apparatus, are likely to be developed further by research groups in the coming years into miniaturised sensor platforms for potential POC applications.

5.2 Acoustic based immunosensors

5.2.1 Quartz crystal microbalance. Quartz crystal microbalance (QCM) sensors have been used in a wide range of sensing applications for a number of years. Initially used in gas sensing, the mass sensitivity of QCMs has recently been utilised for the detection of various biological molecules in an immunosensors format.^{127–130,132,133} A typical QCM sensor comprises a thin quartz crystal placed between two metallic electrodes, which are used to create an alternating electric field across the crystal, as shown in Fig. 13A.¹⁰⁷ The field leads to vibrational motion of the crystal at its resonant frequency, typically in the range of 10–50 MHz.¹³⁵ QCMs are known as bulk wave sensors as the produced propagating acoustic wave travels through the whole of the piezoelectric crystal and can be referred to as bulk acoustic wave (BAW) sensors. The vibrational displacement of the crystal is maximised on the upper and lower surfaces making the crystal sensitive to mass loading. This results in a measurable change in the resonant frequency/resistance of the crystal. More detailed information relating to the various QCM biosensor configurations can be found in the excellent review conducted by Buttry *et al.*¹³¹

Typically QCM immunosensors require suitable modification of the quartz crystals to allow for binding of the target biomarker/molecule. This is achieved by the immobilisation of reaction antibodies onto the QCM surface, which act to capture the target antigen found in a sample placed over the sensor.^{127,128,132} Alternatively, the target antigen can be immobilised onto the sensor area and the mass loading of the crystal can be achieved by binding of complementary antibodies.^{129,133} Reagents are generally delivered and removed from the sensor area by means of microfluidic style flow cells, operating in conjunction with a mechanical pumping system. QCM biosensors have been utilised for the detection of various pathogens and biomarkers, including the detection of the cardiac specific biomarker CRP, with a demonstrated detection limit of the order of pg mL^{-1} .^{129,132} In work by Kurosawa *et al.*,¹³² a QCM immunosensor demonstrated pg mL^{-1} detection of CRP directly from serum samples. This detection sensitivity compares favourably against modern immunosensors formats and demonstrates the potential of QCM sensors for use in medical diagnostics.

A novel immunosensor format, named the latex piezoelectric immunoassay (LPEIA), was demonstrated in work by Aizawa *et al.*¹³⁰ This sensor requires no immobilisation of antibodies or antigens to the surface of the QCM. The sensor operates with reaction antibodies conjugated to 100 nm diameter latex beads which, following the addition of CRP containing serum, forms aggregates consisting of the latex beads and CRP antigen. The QCM responds with a frequency shift upon formation of these aggregates and provides a means of quantifying the biomarker

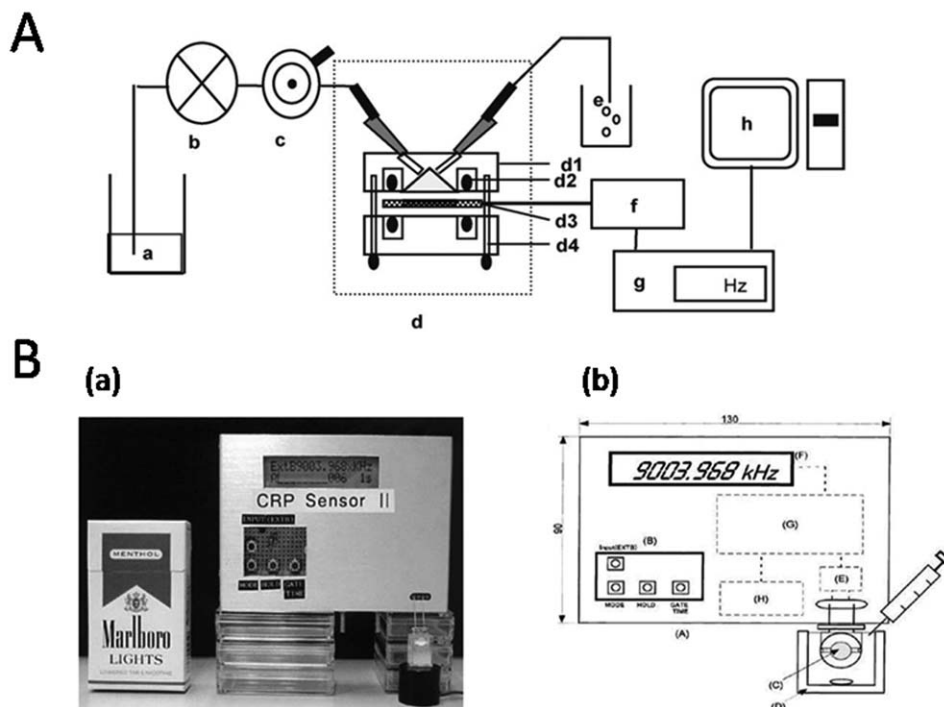


Fig. 13 (A) Schematic of a flow-type QCM immunosensor system as demonstrated by Kim *et al.*¹²⁹ (a) Buffer reservoir, (b) micro-dispensing pump, (c) injector, (d) flow-through cell, (d1) acrylic holder, (d2) O-ring, (d3) QCM, (d4) joint, (e) disposal basin, (f) oscillator module, (g) quartz crystal analyser and (h) PC. © 2009 Elsevier. (B) Diagrams illustrating the QCM immunosensor system as demonstrated by Aizawa *et al.*¹³⁰ (A) photograph of the QCM packaged device. (B) (a,b) schematic of the components comprising the QCM immunosensor, A: body of the CRP sensor, B: switches, C: QCM, D: reaction cell, E: oscillation circuit, F: display, G: frequency counter and H: manganese cell. © 2001 Elsevier.

concentration. The immunosensor, shown in Fig. 13B, was packaged as a portable device, and was capable of performing measurement of CRP within human serum. The device is claimed to be sufficiently sensitive to detect CRP in human serum for clinical analysis ($1 \mu\text{g dL}^{-1}$ LOD), however, the presented results would suggest a detection limit of approximately $10 \mu\text{g dL}^{-1}$.

QCM immunosensors show much promise for the future, owing to their impressive detection sensitivities for a host of target molecules and demonstrated usage with non-ideal samples, such as serum. QCM immunosensors readily lend themselves towards POC applications, as demonstrated in work by Aizawa *et al.* Despite these advantages, a commercial POC QCM immunosensing device has yet to be demonstrated, but could feasibly become a reality as QCM immunosensor research continues.

5.2.2 Surface acoustic wave. As with QCM, surface acoustic wave (SAW) sensor were primarily used as gas sensing devices due to the large attenuation of such devices in liquids. By the late 1980s, various techniques were however developed making SAW devices strong contenders for biosensing applications.¹³⁵ A SAW is a type of acoustic wave that propagates, confined to the surface of a piezoelectric crystal. A typical SAW biosensor operates in a chip based format and consists of a piezocrystal coupled between two interdigitated transducers (IDTs). One IDT is used to convert an electrical signal into a polarised transversal acoustic wave, which propagates across the surface of the piezoelectric crystal and onto the second IDT for detection. Consequently, the surface becomes sensitive to mass loading of the piezoelectric crystal, leading to a shift in the detected

frequency/phase change of the acoustic wave and allows for the crystal surface to act as a biosensor. A conceptualisation of a general SAW immunosensor can be seen in Fig. 14A. Due to the high sensitivity of the SAW sensor to ambient conditions (temperature, turbulence within reaction solution, *etc.*) and because of the subtle variability between individually manufactured SAW sensor components, a typical measurement is performed using two identically configured sensors, one acting as the measurement sensor and the second as a reference sensor.

There are numerous variants of SAW sensors, including Rayleigh-SAW,^{138,141} Lamb-wave Sensors and Love-wave sensors.^{137,140} Love-wave style sensors are however predominantly used due their robustness to liquid based measurements and higher sensitivity.^{134,135} Love waves in SAW devices manifest themselves by the creation of an acoustic resonance of a horizontally polarised shear wave as it travels along the surface of the piezocrystal. The guiding of the acoustic wave is facilitated by the deposition of a layer on the substrate.¹³⁵ Confinement of the acoustic wave to only the guiding layer is highly advantageous as it minimises losses and increases the potential sensitivity of the sensor.

As with QCM sensors, the immunoassay is typically performed on the surface of the crystal and requires suitable modification of the surface for the binding of the biological agents. Reagents are typically delivered and removed by a microfluidic style flow cell operating with a mechanical pumping system. The similarities with QCM continues with the demonstrated immunoassay formats, which typically consist of immobilisation of either antibodies^{138,139,142} or antigens^{137,140} to the surface of the piezocrystal and the measured mass action results from the binding of the corresponding complementary biomolecule. In the work by Lee *et al.*¹⁴⁰ a Love-wave type SAW immunosensor configuration was used for the detection of hepatitis B antibodies, achieving pg mL^{-1} detection limits, demonstrating the high sensitivity of such sensors. The SAW device operated in conjunction with various pumping and control systems integrated into a printed circuit board packaging, as shown in Fig. 14B, and could operate directly using whole blood test samples, demonstrating the potential of SAW biosensors towards POC applications.

A unique SAW immunosensor format has been demonstrated in the work by Fertier *et al.*,¹³⁶ which consisted of the immobilisation of peptides onto a semicarbazide functionalised quartz piezocrystal surface. The peptides would bind to complementary sites on target antibodies and demonstrated low $\mu\text{g mL}^{-1}$ detection limits of anti-HA murine antibodies. The devised SAW sensor showed no appreciable non-specific binding and shows that peptide binding may provide a novel alternative to the use of immobilised complementary antigens in SAW immunosensors. Target molecules need not only be restricted to immobilisation upon the piezocrystal surface and, in the work by Stubbs *et al.*,¹⁴¹ a two port SAW resonator immunosensor was demonstrated with antibodies immobilised onto the metal electrodes of the device. The immunosensor architecture was also altered such that reflection gratings were integrated at either extreme of the sensor where, together with the IDTs, they formed a resonant acoustic cavity. This sensor was primarily intended as a vapour-phase sensor and no demonstration of the detection capabilities was reported.

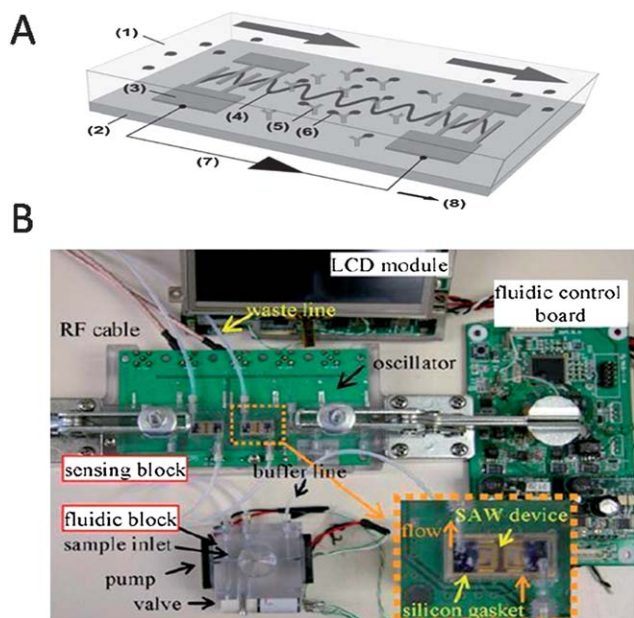


Fig. 14 (A) Schematic of general SAW immunosensor, adapted from Lange *et al.*¹³⁵ (1) liquid sample in which the immunosensor is immersed (top arrows indicate fluidic flow), (2) piezoelectric crystal, (3) IDT's, (4) surface acoustic wave, (5) immobilised antibodies, (6) target analyte molecules, (7) driving electronics and (8) output signal.  2008 Springer-Verlag. (B) Photograph of the SAW based immunosensing system as demonstrated by Lee *et al.*¹⁴⁰  2009 Elsevier.

Very little literature is available with respect to the detection of cardiac specific biomarkers using SAW immunosensors. This may be due to a number of patents that have been filed surrounding the usage of SAW sensors for the detection of prominent cardiac markers such as cTnI, cTnT, myoglobin and CK-MB.^{144,145} In the work by Chang *et al.*,¹⁴⁶ myoglobin was immobilised onto a C60-protein functionalised SAW sensor and used for the detection of anti-myoglobin antibodies. The immunosensor was demonstrated in a multiplexed, dual SAW crystal configuration such that one crystal was functionalised with myoglobin antigen and the second with haemoglobin molecules. The sensor showed good selectivity and detection sensitivity for the examination of a mixture containing anti-myoglobin ($0.035 \mu\text{g mL}^{-1}$ LOD) and anti-haemoglobin ($0.32 \mu\text{g mL}^{-1}$ LOD) antibodies and demonstrates the multiplexing potential of SAW immunosensors.

SAW and QCM immunosensors share many similarities in terms of device usage, principles of immunoassay reactions and demonstrated detection sensitivities. There appears however to be much more research interest in the usage of QCM over SAW sensors, possibly due to the added design complexity of the integrated IDT around the piezocrystal substrate. Love-wave SAW sensors may however offer advantages over the use of QCM due to the nature of the propagating acoustic wave travelling through a restricted surface layer and not through the bulk of the crystal, resulting in reduced energy losses and a potentially increased sensitivity over the sensing surface. Many research groups are currently developing sensing platforms that reduce the size of a packaged sensor and the required assisting

equipment,^{136,140,141,143} making SAW sensors more desirable for POC applications. In light of the demonstrations of SAW sensors for use with blood samples^{140,142} a portable POC SAW based system may be achievable, although not yet demonstrated.

6 Multiplexing

Fluorescence intensity immunoassay biosensors can be readily applied to multiplexing applications by spatially addressing the biosensor with antibodies specific to a particular biomarker.^{19–21} This multiplexing can take the form either of a microarray, or by spectrally resolving the signal from unique fluorescent tags.^{18,28,36} Chemiluminometric based multiplexed biosensors have yet to be demonstrated. In the work by Plowman *et al.*,¹⁹ the simultaneous excitation of multiple sensor areas was addressed using a SiON optical waveguide to act as the immobilisation substrate for the reaction antibodies. A laser excitation source was focused down the length of the waveguide allowing for the excitation of fluorescently labelled antibodies on three spatially addressed sensor areas. This methodology successfully detected three independent cardiac markers (cTnI, myoglobin and CK-MB) at clinically relevant concentrations. A microarray style immunoassay biosensor, similar to those regularly utilised for DNA analysis, has been demonstrated in the work of Gul *et al.*²⁰ The microarray was constructed simply by the printing of immobilisation antibodies onto the surface of a glass microscope slide and optical measurements of excited fluorescent labels were carried out using a benchtop biochip reader. Numerous protein markers were investigated, including the cardiac biomarkers myoglobin and

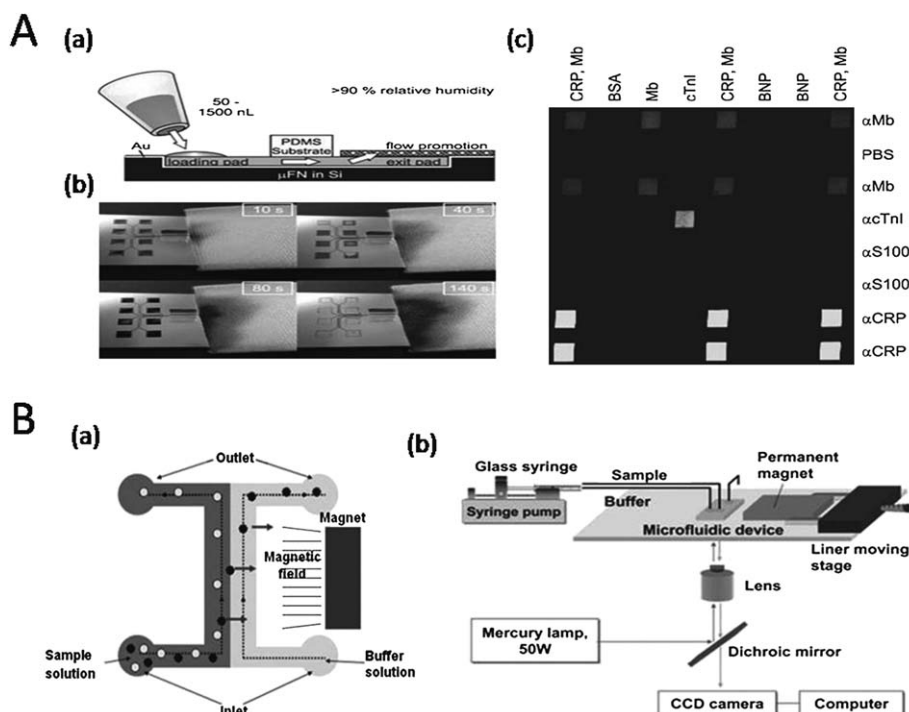


Fig. 15 (A) Diagrams detailing the device and corresponding results from various multiplexed based immunosensing platforms as demonstrated by Wolf *et al.*²¹ (a) A side profile of a microfluidic loading port. (b) Photographs of a multiplexed immunosensor during fluidic flow promotion at various time intervals. (c) Imaging results showing micromosaic binding events on the sensor surface. © 2003 Elsevier. (B) Diagrams illustrating various aspects of the magnetic force based PMP immunosensor, adapted from Kim *et al.*¹¹⁵ (a) Figure illustrating the magnetic force based detection principle. (b) Diagram of the experimental apparatus utilised in conjunction with the microfluidic chip device. © 2005 The Royal Society of Chemistry.

CRP, with results comparable to those from three independent commercial immunoassay kits. This work demonstrates the versatility of the immunoassay format on a simple substrate. This format could readily be adapted for use in a POC device, while still achieving a high level of sensitivity. Multiplexed detection has been achieved in work by Caulum *et al.*¹⁸ by the use of cleavable immunotags. In this format four independent tags are cleaved from a sandwich structure and are separated by means of micellar electrokinetic chromatography (MEKC). The biosensor signal is not imaged on the surface of sensor substrate in this novel method. Individual labels are determined by their unique migration time, a method which is reminiscent of capillary electrophoresis. This technique has been used in determining the concentration in serum samples of four cardiac specific markers myoglobin, CK-MB, cTnI and cTnT with detection limits from ng mL⁻¹ down to pg mL⁻¹. Possible drawbacks to this technique are the reasonably long assay times compared with the spatially addressed intensity measurements, due primarily to the additional reaction stages following the sandwich structure formation. In addition, the cost per assay is also higher due to the requirement for the cleavage solution.

Many promising chip based multiplex microfluidic biosensor platforms have been demonstrated by researchers at IBM. Various devices and results are presented in Fig. 15A. The most impressive of these platforms, referred to as the micromosaic immunoassay, allows for the parallel delivery of a sample to multiple sensor areas.^{21,22,24} The biosensor area consists of reaction antibodies immobilised onto the PDMS surface that act to capture the target biomarker, forming a sandwich structure with a fluorophore labelled secondary antibody. The micromosaic device has been demonstrated for the simultaneous detection of numerous cardiac markers, such as cTnI, CRP, myoglobin, in spiked serum samples with ng mL⁻¹ detection sensitivities.

QD based biosensors also hold considerable promise for multiplexed immunoassay reactions. In the work by Goldman *et al.*,^{34,36} four independent QDs of emission maxima at 510, 555, 590 and 610 nm, were conjugated with antibodies to perform a sandwich style immunoassay for the detection of cholera toxin, ricin, shiga-like toxin 1 and straphylococcal enterotoxin B. Using a single excitation source, a commercial plate reader and a deconvolution algorithm, all four QD signals were successfully recovered for examined toxin concentrations of 1000 and 30 ng mL⁻¹. Such multiplexing using a single excitation source would not be possible using organic dyes unless multiple excitation sources, high performance emission filters and complex data analysis were to be used to recover the respective contribution of each dye from the final detectable signal. A recent review by Sapsford *et al.*²⁶ commented however that the single major obstacle that hinders the multiplexing of over 6 individual QDs is the cross reactivity of the conjugated antibodies.

Plasmonic based sensor technology has been applied to the multiplex detection of cardiac specific biomarkers. In work by Endo *et al.*, a chip based protein array format was created by spotting detection antibodies onto a modified gold SPR sensor.⁶⁷ The sensor was fabricated on gold coated glass substrate and comprised of a self assembled monolayer of silica microparticles, where upon monolayer formation; a gold layer was deposited over the resulting structure. The protein array was tested for the

simultaneous concentration measurements of immunoglobulin A (IgA), IgD, IgG, IgM, CRP and fibrinogen, achieving detection limits of 100 pg mL⁻¹ for all target analytes.

SERS based biosensors have been proven to have the capacity for multiplexing, though the use of multiple unique Raman reporter labels (thiophenol (TP), 2-naphthalenethiol (NT), *etc.*) to metallic nanoparticles. One of the earliest demonstrations of this immunosensing format was by Ni *et al.*,⁷⁸ where simultaneous immunoassays for two reporter labelled gold colloids were discriminated within a single sensor area with a 30 ng mL⁻¹ limit of detection. This assay format was further demonstrated in work by Grubisha *et al.*,⁸⁴ for the detection of prostate specific antigen (PSA), yielding impressive detection limits of 4 pg mL⁻¹. Despite Grubisha *et al.* describing the multiplexed assay concept it was not demonstrated beyond the detection of a single target.

An interesting PMP based multiplexed immunosensor has been demonstrated in the work by Kim *et al.*,¹¹⁵ based upon the measurement of the target analyte concentration resulting from the deflection caused by the influence of a magnetic field upon fluorescent polymer microbead conjugated PMPs, as shown in Fig. 15B. Despite this immunosensing format not being applied to cardiac marker detection, the methodology demonstrates a novel detection methodology based on particle separation. The sensor uses a sandwich style immunoassay, with the PMP acting as the immobilisation substrate for the capture antibodies and with secondary antibodies labelled with fluorescent microbeads. Following capture of the target antigen, the imaging of the microbead allows for the velocity of the conjugate to be determined, which is proportional to the total volume of conjugated PMPs, which, in turn, depends on the concentration of the captured target antigen. The device was demonstrated for the detection of rabbit IgG (44 pg mL⁻¹ LOD) and mouse IgG (15.6 ng mL⁻¹ LOD). In the same work a multiplexed, dual analyte detection assay was performed on the device using two fluorescent microbeads, each specific to one particular target antigen. Only qualitative results were presented, however, the device was capable of discriminating the two individually labelled PMP conjugates. This type of immunosensor may however be unsuitable for POC applications due to the complexity of the system which requires the use of dual syringe pumps, optical excitation and detection apparatus, a permanent magnet rig, added to cumbersome data analysis. Further, the use of a fluorescent label and PMPs would also increase the cost per assay compared to alternative magneto-immunosensors.

A multiplexed MC immunosensor has been demonstrated in the work by Arntz *et al.*¹²² for the detection of myoglobin and CKMB, achieving $\mu\text{g mL}^{-1}$ limits of detection using a laser deflection configuration. This sensor appears to be one of the only demonstrations of a multiplexed MC immunosensor but suffers from low detection sensitivities of less than 20 $\mu\text{g mL}^{-1}$, when compared with piezoelectric based MC immunosensors, which have a demonstrated detection sensitivity of 1 ng mL⁻¹ for myoglobin samples.¹²⁰

Multiplexed biosensors have predominantly been demonstrated as fluorescence intensity based immunosensing formats, owing to their high detection sensitivities and proven adequacy in DNA microarray based technology. Alternative, multiplexing capable, immunosensing formats have been demonstrated in the

literature, with SPR and SERS based strategies being the most successful, yielding detection sensitivities comparable to fluorescence intensity based devices. Typically, multiplexed based systems are not designed with the intension of serving as POC platforms, as generally a complex and bulky detection set-up is required to individually address each detection signal. Realistically, for POC diagnostics only a small number of biomarkers are likely to be examined and so array style multiplexing becomes redundant.

7 Conclusions

The challenges associated with the integration of immunosensor technology into a clinical setting are multifaceted in nature. For a particular biosensor system to become part of routine medical use, it is paramount that the sensor is tailored to meet the demands of the clinicians, thus increasing the potential degree of usefulness, acceptance and utility of such an instrument. In terms of future development for POC applications it would be desirable for a biosensor device to be a cost effective, disposable device that encapsulates all the required instrumentation, in a suitably portable format. Given the maturity and proficiency of most immunosensor technologies, research focus has shifted towards

the suitable miniaturisation of reagent delivery systems and excitation/detection apparatus, while additionally reducing overall power requirements.

Each demonstrated biosensor technology has its own inherent advantages and disadvantages to usage and no single format has emerged as the dominant technology. A summary of the most capable biosensors demonstrated for the detection of various cardiac markers can be found in Table 3. This summary includes a ranking of each demonstrated formats potential for development as a POC capable diagnostics instrument based upon the portability of the biosensor and the potential for reducing the number of assisting devices and detection apparatus. It is observed in Table 3 that the more promising biosensor formats which can be readily applied towards POC applications include electrochemical, chemiluminescent and fluorescence intensity measurements, each of which are capable of detection of cardiac markers at clinically relevant sensitivities (ng mL^{-1} to pg mL^{-1}) and have been demonstrated using unconditioned, blood samples. It is therefore unsurprising that such biosensors formats are utilised in the handful of commercially available POC immunosensor instruments capable of cardiac marker detection as described in Table 1.

Table 3 State of the art biosensors demonstrated for the detection of cardiac biomarkers

Detected Marker	Biosensor Type	Reference	Limit of detection	Assay TAT	POC Potential (High [1]–Low [10])
Troponin I	Fluorescence Intensity	Caulum <i>et al.</i> 2007 ¹⁸	2 ng mL ⁻¹	> 4.5 h	4
	Chemiluminescence	Cho <i>et al.</i> 2009 ⁴⁵	0.1 ng mL ⁻¹	~23 min	2
	Quantum Dot	Stringer <i>et al.</i> 2008 ²⁹	55 nM	1–10 min	6
	FRET	Grant <i>et al.</i> 2005 ³⁹	94 nM	Not stated	6
	SPR	Masson <i>et al.</i> 2004 ⁵⁹	1.4 ng mL ⁻¹	>10 min	6
	Potentiometric	Purvis <i>et al.</i> 2003 ¹⁰⁰	0.01 ng mL ⁻¹	15 min	2
	PMP	Kiely <i>et al.</i> 2007 ¹¹²	0.5 ng mL ⁻¹	~4 min	4
Troponin T	Fluorescence Intensity	Caulum <i>et al.</i> 2007 ¹⁸	0.025 ng mL ⁻¹	>4.5 h	4
	FRET	Mayilo <i>et al.</i> 2009 ⁴²	0.7 ng mL ⁻¹	Not stated	6
CRP	Fluorescence Intensity	Gervais <i>et al.</i> 2009 ²⁵	1 ng mL ⁻¹	~14 min	3
	Chemiluminescence	Bhattacharyya <i>et al.</i> 2007 ⁴⁷	100 ng mL ⁻¹	25 min	9
	ECL	Miao <i>et al.</i> 2004 ⁵³	10 ng mL ⁻¹	>3 h	5
	SPR	Endo <i>et al.</i> 2006 ⁶⁷	0.1 ng mL ⁻¹	>30 min	7
	SERRS	Campbell <i>et al.</i> 2008 ⁸⁰	0.3 ng mL ⁻¹	Not stated	9
	Impedimetric	Chen <i>et al.</i> 2008 ¹⁰⁶	0.1 ng mL ⁻¹	60 min	8
	PMP	Luxton <i>et al.</i> 2004 ¹¹³	3 ng mL ⁻¹	>3 min	6
	Microcantilever	Lee <i>et al.</i> 2004 ¹²³	10% of unknown conc.	60 min	8
	QCM	Kurosawa <i>et al.</i> 2004 ¹³²	0.001 ng mL ⁻¹	90 min	7
Myoglobin	Fluorescence Intensity	Gul <i>et al.</i> 2007 ²⁰	6.5 ng mL ⁻¹	30 min	7
	Chemiluminescence	Darain <i>et al.</i> 2009 ⁴⁶	16 ng mL ⁻¹	Not stated	6
	SPR	Masson <i>et al.</i> 2004 ⁵⁹	2.9 ng mL ⁻¹	>10 min	6
	MEF	Matveeva <i>et al.</i> 2007 ⁷³	>100 ng mL ⁻¹	1 h	6
	SERRS	Bizzarri <i>et al.</i> 2002 ⁸⁹	Single molecule detection	Not stated	9
	Impedimetric	Billah <i>et al.</i> 2008 ⁹⁴	15 ng mL ⁻¹	~80 min	5
	Potentiometric	Wang <i>et al.</i> 2008 ⁹⁶	1000 ng mL ⁻¹	2–10 min	7
	Aperometric	O'Regan <i>et al.</i> 2002 ¹⁰¹	80 ng mL ⁻¹	30 min	5
	Microcantilever	Kang <i>et al.</i> 2006 ¹²⁰	10 ng mL ⁻¹	Not stated	7
	SAW	Chang <i>et al.</i> 2007 ¹⁴⁶	35 ng mL ⁻¹	>10 min	8
CKMB	Fluorescence Intensity	Caulum <i>et al.</i> 2007 ¹⁸	3 ng mL ⁻¹	>4.5 h	4
	PMP	Luxton <i>et al.</i> 2004 ¹¹³	2 ng mL ⁻¹	>3 min	6
	Microcantilever	Arntz <i>et al.</i> 2003 ¹²²	20 000 nL mL ⁻¹	>10 min	9
BNP	SPR	Kurita <i>et al.</i> 2006 ⁶¹	0.005 ng mL ⁻¹	5–15 min	3
H-FABP	Amperometric	O'Regan <i>et al.</i> 2002 ¹⁰¹	4 ng mL ⁻¹	50 min	7

An unavoidable detection sensitivity limiting factor experienced by all commercial and research grade cardiac biomarker detection instruments is due to the reactivity of the selected antibody or antibody pair. The selectivity between antibodies can dramatically alter due to the selected antigen-antibody epitope target site, the pH and salt concentration of the surrounding environment, whether they are mono or polyclonal, *etc.* It is observed that no single set of antibodies are considered the 'ideal' antibodies for a given biosensing application or cardiac biomarker target and so in general multiple antibodies would need to be examined and optimised for a specific set of reaction conditions. Additionally, the cross-reactivity of selected antibodies would have to be assessed in terms of the undesired binding events occurring with alternative proteins found within an examined test samples. Cross-reactivity is therefore a non-trivial and unavoidable sensitivity limiting factor, which can be particularly problematic at the typically low concentrations utilised within LoAC devices. In terms of immunosensing technology, methodologies that utilise a single recognition phase (antibody-antigen complex) suffer reduced specificity compared to dual recognition phase (sandwich structure) strategies. Conversely, dual recognition bioassays suffer twice the potential statistical variations resulting from antibody-antigen binding events, potentially limiting immunoassay sensitivity and repeatability. Most of the commercially successful POC cardiac biomarker detection devices (Table 1) utilise a dual recognition phase strategy, possibly because the high level of specificity of this immuno-format ultimately results in reduced false positives, a feature highly desirable for medical device.

Many high sensitivity optical techniques, such as SPR or SERS based detection, despite their applicability for targeted cardiac biomarker detection, are hindered by the size and complexity of the required excitation and detection apparatus. Similarly, many optical intensity based LoAC devices are currently hindered for quantitative analysis by the requirement of microscopes to adequately image the sensor area. It is worth noting that a handful of commercial PoC cardiac marker detection devices do contain suitably miniaturised fluorescence/chemiluminescence detection apparatus (Table 1). Additionally, numerous portable fluorescence detection systems are readily available from a wide variety of biotechnology and optoelectronics companies and have been demonstrated successfully for DNA based bioanalysis systems.^{43,44,57} Despite this wealth of technology the challenge remains for researchers to adequately adapt the existing LoAC cardiac biomarker based analysis systems to incorporate such instruments. This could potentially be a motivating factor behind the interest in non-optical based techniques, as it generally negates the requirement for bulky apparatus.

Lateral flow based devices, comprising of a series of membrane, absorbent and conjugation pads, can arguably be considered the first generation of LoAC based immunosensing devices. Primarily, they operate to provide automated fluidic delivery of self-contained reagents to a biosensing area in a neatly packaged, miniaturised device. Lateral flow based diagnostics kits are a very mature technology that have seen wide commercial development and success in terms of cardiac biomarker detection and the now widely recognised home pregnancy kit. Predominantly, cardiac biomarker lateral flow devices are qualitative in nature (Cardiac STATus, Nanogen), operating by

a visible chemiluminometric based confirmatory signal above a pre-determined clinical cut-off concentration. More modern commercial lateral flow devices (Cobas h232, Roche Diagnostics Ltd) have seen the integration of portable optical detection instrumentation making such devices a more quantitative cardiac biomarker evaluation tool. The natural fluidic actuation of lateral flow devices resulting from the natural capillary systems of the absorbing pads is highly advantageous as an automated diagnostics platform leading to continued interest from various research groups. More recent platforms have demonstrated increased detection limits compared to their first generation counterparts by the use of gold nanoparticle technology. Given that lateral flow devices can be used directly with blood samples, it is anticipated that as the immunosensing technology matures, with the likely conjugation of modern nanoparticle technology, further, more sensitive devices will be created in the coming years.

The second generation of LoAC devices utilise the benefits of on-chip microfluidic networks and are considered the leading candidate technology for the next generation of portable cardiac marker detection devices, due to their potential to operate in a fully or near fully automated fashion.^{21–25} A POC based cardiac marker detection device would ideally be expected to operate directly from a patient's blood sample. It has been demonstrated that this is possible for a range of modern biosensing platforms while still demonstrating reasonably high detection sensitivities. Issues relating to non-specific binding of other proteins found within a blood sample will ultimately restrict the sensitivity and reproducibility of a given immunosensor and so sample pre-treatment may be required to overcome this potential bottleneck. To date, few solutions have been presented to this challenge. Ironically one of the few presented solutions, as demonstrated by Gervais *et al.*, saw the use of a membrane based absorbing pad as a blood filtration element.²⁵ This is essentially a component from a lateral flow based device and demonstrates how solutions to non trial issues relating to LoAC bioanalysis could readily be found through the conjugation of existing technologies. A further challenge to the overall construction of a POC enabling LoAC device is a means for automated fluidic flow. Most microfluidic based method of sample/reagent delivery are based upon actuated fluidic flow initiation (mechanical pumps, direct syringing, *etc.*), making the overall device impractical for POC use. Such limitations are not experienced by lateral flow devices, which utilise the natural capillary systems of the absorbent pad with which to initiate and maintain fluidic flow. Solutions within microfluidic systems to this issue have elegantly been demonstrated by various groups, by which manufactured capillary networks initiate and maintain fluidic flow within microfluidic networks.^{23–25} It is therefore anticipated that future POC cardiac biomarker detection devices are likely to be a conjugation of LoAC technologies that enhance device automation, portability (including the detection apparatus), detection sensitivity (within/beyond current clinical cut-offs) and the necessary multiplexing capabilities.

This review has hoped to demonstrate that the field of cardiac biomarker technology is a highly researched, yet highly evolving and adaptable field with much more potential scope for further novel sensor integrated LoAC formats to be demonstrated. In order to realise such expectations and to improve current immunosensor technology, the task will involve not only

innovation on the part of the engineers examining new sensor technologies and material, but also on the part of chemist and biologist in creating new immunoreactions formats, while maximising reaction efficiencies and applicability for biosensing purposes. Immunosensor technology is at the heart of targeted cardiac biomarker detection and has allowed for clinically applicable detection sensitivities from a variety of novel detection formats. Such technology, enabled for practical applications by microfluidic and LoAC platforms, is not only applicable to cardiac biomarker diagnostics but can also be utilised towards the detection of alternative clinically relevant biomarkers and molecules, added to a host of non-clinical biological applications such as environmental monitoring or food science studies.

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