Review Article

Real-time and Label-free Bio-sensing of Molecular Interactions by Surface Plasmon Resonance: A Laboratory Medicine Perspective

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Keywords
surface plasmon resonance, biosensor, real-time, label-free.

Abbreviations
SPR, surface plasmon resonance; CFCA, calibration-free concentration analysis; LOC, lab-on-a-chip; POCT, point-of-care testing; INR, international normalized ratio; SNP, single nucleotide polymorphisms; BioMEMS, bio-micro-electro-mechanical-system; SPRi, surface plasmon resonance imaging; SERS, surface enhanced Raman scattering.

Abstract
Radioactive, chromogenic, fluorescent and other labels have long provided the basis of detection systems for biomolecular interactions including immunoassays and receptor binding studies. However there has been unprecedented growth in a number of powerful label free biosensor technologies over the last decade. While largely at the proof-of-concept stage in terms of clinical applications, the development of more accessible platforms may see surface plasmon resonance (SPR) emerge as one of the most powerful optical detection platforms for the real-time monitoring of biomolecular interactions in a label-free environment.

In this review, we provide an overview of SPR principles and current and future capabilities in a diagnostic context, including its application for monitoring a wide range of molecular markers of disease. The advantages and pitfalls of using SPR to study biomolecular interactions are discussed, with particular emphasis on its potential to differentiate subspecies of analytes and the inherent ability for quantitation through calibration-free concentration analysis (CFCA). In addition, recent advances in multiplex applications, high throughput arrays, miniaturisation, and enhancements using noble metal nanoparticles that promise unprecedented sensitivity to the level of single molecule detection, are discussed.

In summary, while SPR is not a new technique, technological advances may see SPR quickly emerge as a highly powerful technology, enabling rapid and routine analysis of molecular interactions for a diverse range of targets, including those with clinical applicability. As the technology produces data quickly, in real-time and in a label-free environment, it may well have a significant presence in future developments in lab-on-a-chip technologies including point-of-care devices and personalised medicine.

Introduction

The growth in biosensor devices that use electrochemical, optical, piezoelectric and thermometric methods to monitor real-time, highly specific, biomolecular recognition events has increased rapidly over the last 20 years.[1] Indeed, over 10,000 journal articles with the term "biosensor" were published by 2010 (Figure 1). The exponential rate of growth over this time and the rapid uptake of biosensor applications for lab-on-a-chip (LOC) and point-of-care testing (POCT) indicates that rapid developments in the broad field of biosensors will have an enormous and increasing impact on all our health care in the future.

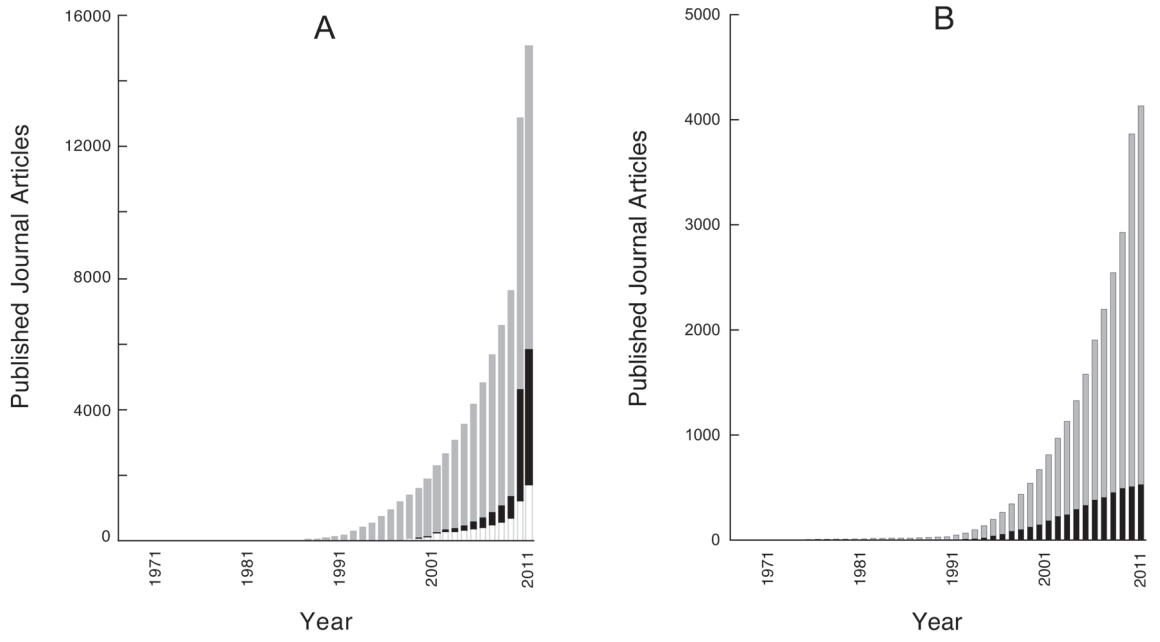

Figure 1. Growth in biosensor and SPR publications. Total number of journal articles published up to a given year with: (A) "Biosensor" (grey columns), "Lab-on-a-chip" and "biochip (black columns), and point-of-care testing (open columns) in the article title for all journals; and (B) "BIAcore", "ProteON" or "surface plasmon resonance" in the article title for all journals (grey columns) including those with biochemistry clinical and medical content (black columns) from 1967 to 2011, inclusive. Data collected using the advanced search functions within the http://www.scirus.com/ web search engine.

Surface Plasmon Resonance (SPR) technology is an increasingly accessible optical detection method for biosensors. The importance of the technology and its application is evident from the accelerating rate of growth in journal articles published in this area, which mirrors the growth in biosensor research (Figure 1). The application of SPR to the clinical chemistry and medical sectors will gain momentum as the technology is understood and embraced within these sectors. There are already many exciting developments of the technology and its applications.

The purpose of this review is to introduce SPR concepts and technology to the reader and to discuss applications with the potential to influence the way we conduct clinical chemistry and laboratory testing in the future.

Principles and Operational Parameters of SPR Instruments

In 1968, Otto reported the excitation of non-radiative surface plasma waves in silver by the method of frustrated total reflection.[2] Fifteen years later, Lieberg described the use of surface plasmon resonance for gas detection and biosensing.[3] However, SPR was not available commercially for biomolecular interactions until 1990 when the first SPR instrument was released onto the market.[4] Today, a number of companies provide SPR biosensor capabilities (Table 1).

Typical SPR instrumentation comprises a monochromatic polarised light source, a glass prism, a thin metal film in contact with the prism base, and a photodetector.[5] Recognised as prism-coupled SPR, this design is the most commonly used platform for SPR instrumentation. Other systems designs typically use either waveguide or grating coupled SPR.[6,7] By passing polarised light through a high refractive index prism, the polarised light becomes totally internally reflected (TIR) and generates an evanescent wave that penetrates the thin metal film (Figure 2). Monitoring the intensity of the reflected light as a function of the angle of incidence, the intensity of reflected light will pass through a minimum, as energy from the evanescent wave is absorbed by the surface plasmons in the thin metal film. As the polarised light is capable of exciting the surface plasmons, the photons of light will interact and induce oscillations with the surface plasmons of the metal film, and this is reflected as a decrease in the intensity of the reflected light.[8] The angle at which the reflected light intensity occurs is known as the SPR angle (Ø). The SPR angle is sensitive to changes in the dielectric refractive index as the field of the electromagnetic waves from excited surface

Table 1. Some commercial SPR products in the market place.

Company*	Product	Market
BioNavis	SPR-Navi	Chemistry
BioRAD	Proteon XPR36	Life Sciences
Biosensing Instrument	BI-2000/BI-3000	Chemistry
GE Healthcare	Biacore	Life Sciences
ICx Nomadics	SensiQ	Life Sciences
Metrohm	Autolab	Chemisty
Plexera Bioscience	Plex Array System	Life Sciences
Reichert/Xantec	SR7000DC	Life Sciences
Sierra Sensors	SPR-2	Life Sciences

*there are also several SPRi systems available not listed here, which are imaging systems that are generally used in-conjunction with a spotter.

plasmons is vastly concentrated in the dielectric medium opposing the prism face. These surface plasmon waves are extremely sensitive to changes in the refractive index at close proximity to the metal surface.[8,9] By the immobilisation of receptor molecules at the metal surface, this technique can be applied to diagnostic applications as the interaction of biomolecules will produce a change in the refractive index near the metal surface, causing a shift in the SPR angle and providing a detectable signal (Figure 2). A number of reviews are also available that further detail the principles of SPR and the associated technology.[10,11]

The capability of SPR instruments is built around a sensor chip, typically a gold-coated glass side that is placed on the prism base with the coating facing upward. To assist in the immobilisation of a ligand of interest (e.g. insulin, if studying interactions with the insulin receptor) is derivatised to a polymer matrix (Figure 2). It should be noted that the field of SPR has its own terminology and for the purposes of this review, the term "ligand" refers to the substance immobilised at the sensor surface, whereas the term analyte refers to the substance in the fluid phase that is passed over the sensor surface and interacts with the "ligand".

A wide range of biomolecules can be immobilised on the surface of a sensor chip using a variety of chemistries including amino, thiol, hydroxyl, carboxyl, and aldehyde group coupling. A common approach is to chemically couple an antibody to the sensor surface, which can then capture its specific antigen. The antibody-captured antigen in turn can be used to capture any of its own specific binding partners. Other capture surfaces include biotin, histidine-tagged and glutathione-S-transferase fusion proteins. Lipid vesicles or subcellular preparations can also be captured so that lipid composition and molecular recognition events between proteins and glycolipids can be directly studied in real-time.

Once the ligand is attached to the sensor chip matrix, solutions can be injected or passed through the polmer matrix to study interactions between the immobilised ligand and analyte(s) in the sample. An example of real-time-derived response data for such a situation is illustrated in Figure 2. The data output is typically referred to as a sensorgram. With reference to Figure 2, a stable baseline is initially observed prior to the injection of the analyte. Upon exposure of the analyte to the surface ligand, an association phase indicative of complex formation is observed. There is a gradual increase to equilibrium between the bound and free analyte, and this is observed as a plateau in the response intensity. To monitor dissociation of the bound analyte, the instrument switches to injecting running buffer. The dissociation phase is representative of the stability of the complex. Often the complex will not completely dissociate and the remaining analyte-ligand complexes can require mild acidic or basic washing conditions to regenerate the surface. This is known as regeneration, and the ligand-derivatised matrix on the chips can be used repeatedly using relatively mild "regeneration" conditions to remove bound analytes between assays or experiments. The gold sensor chips can also be easily reconditioned (the entire protocol can be easily completed within one day) and reused repeatedly by removing the immobilised ligand with a combination of enzymatic (Pronase E) and/or simple chemical treatments.[12,13]

Whilst providing many advantages, SPR studies enable a diverse array of applications to be applied to a broad range of biological targets (Tables 2 & 3) without the need for any radioactive or fluorescent labels. Data is collected continuously (typically, every 0.1 second) over time, and

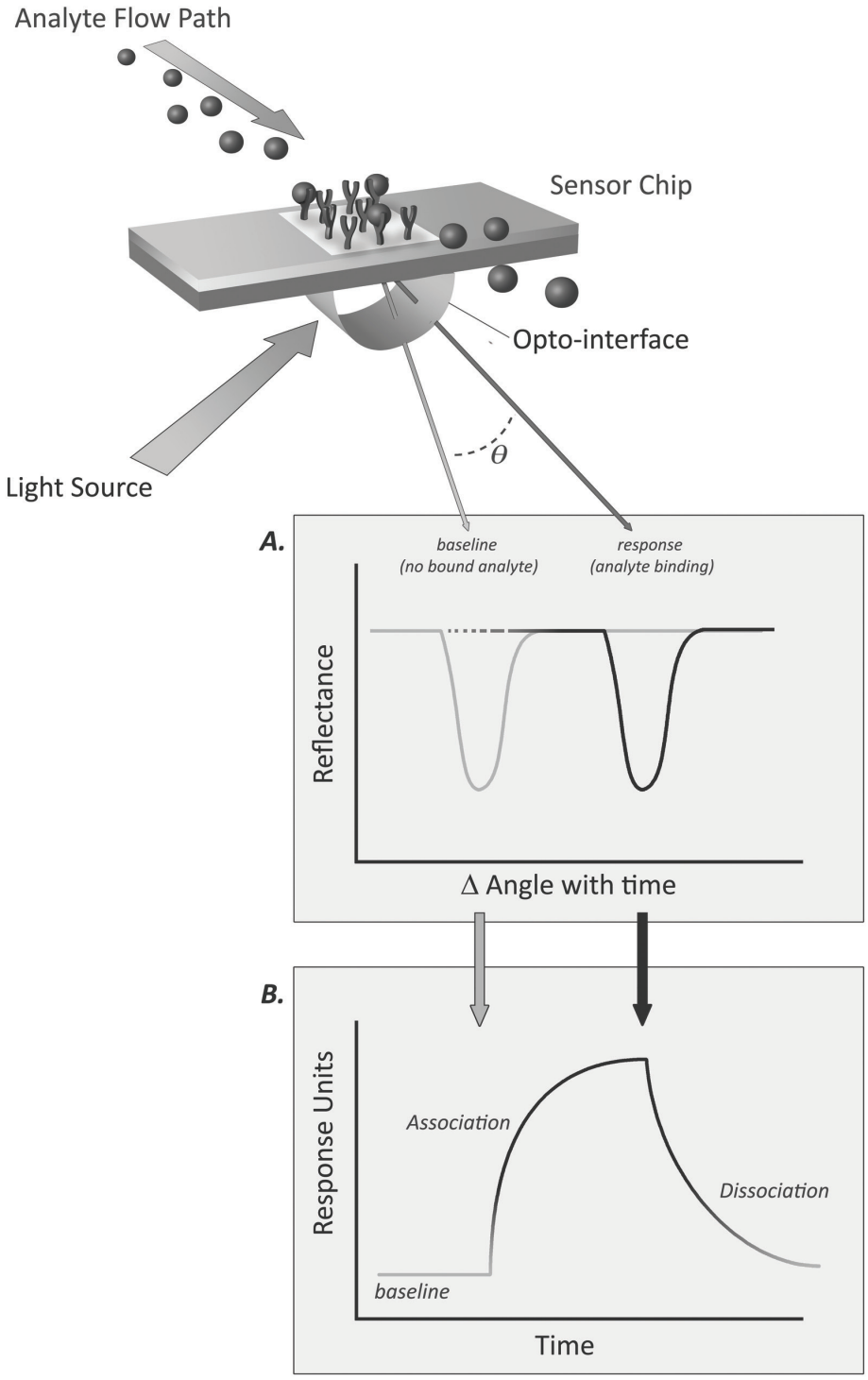

Figure 2. Principle of SPR illustrated. The ligand of interest is attached to a polymer matrix using one of several well-established surface chemistries. The analyte is then passed through a flow cell over the ligand-derivatised matrix. Any change in mass following the interaction between the ligand and analyte is detected as a change in the angle of the incident light needed to generate the surface plasmon resonance phenomenon at the gold polymer interface. This is measured as an energy or reflectance dip as a function of pixels, which translates to response units (RU) over time. The RU change is directly proportional to molecular mass change and so binding kinetics and stoichiometry can be measured in real-time without any label.

Table 2. Applications of SPR.

Targets	Application Examples
> Small molecules > Proteins > DNA and RNA > Glycans > Lipids > Membranes > Cells	• Small molecule screening • Epitope Mapping • Ligand Fishing • Ligand-receptor interactions • Protein-protein, protein-DNA, DNA-DNA • Protein-carbohydrate interactions • Antibody engineering • Antigen antibody Interactions • Vaccine development • Drug discovery • Determine the "active" concentration • Interactions with cell membranes • Interactions with lipid vesicles • Signal transduction cascades • Virus research • Assembly of membrane-bound quaternary signal transduction complex • Compare mutation activities
Evaluates	
o Kinetics (k_{on}; k_{off}) o Affinity (K_D) o Stoichiometry o Thermodynamics o Active analyte concentration o Calibration-free Concentration Analysis (CFCA)	

so the kinetic parameters and binding models can be fitted and compared with confidence and precision. The extremes of kinetic behavior describing low to high affinity molecular interactions can be examined and span association on-rates of 10^3 to 10^9 $M^{-1}s^{-1}$, dissociation off-rates from 10^{-5} to 1 s^{-1}; K_D from μM to pM. If applicable, kinetics are typically determined using a range of sample concentrations, each run in a separate run or cycle (multi-cycle kinetics). Analytes bound to the solid-phase ligand are removed by regeneration of the surface between each cycle. However in some systems, analyte can be injected with increasing concentrations in a single cycle obviating the need for sensor chip regeneration between sample injections. This innovative approach reduces assay development time, provides faster sample throughput, and enables kinetic analysis of molecular interactions that may otherwise be confounded by potentially harsh regeneration conditions between cycles. The real-time binding of beta-2-microglobulin to immobilised anti-beta-2-microglobulin is illustrated in Figure 3 using single cycle and multi-cycle configurations. For this example, we completed a single cycle kinetic approach in one run within 25 minutes, whereas the multi-cycle kinetic approach took several hours to complete. In each case, the data appear to fit a 1:1 binding model.

In addition to detailing binding kinetics, the stoichiometry of interactions can be calculated from the proportionate increase in signal as the analyte binds relative to the known molecular mass of each of the interacting molecules. This is possible because the SPR signal is directly proportional to the mass change at the surface of the sensor chip. Some SPR instruments enable analysis at different temperatures (4-45ºC) allowing determination of thermodynamic contributions of enthalpy and entropy for the molecular interactions. Runs can be completed in minutes using microlitres of highly diluted, precious samples (20-50 μL of >10 pM, depending on instrument). Instrument sensitivity is exceptional (Refractive Index changes $<10^{-5}$). Indeed, with some SPR instruments, there is now no lower limit on the size of the molecules being detected in a binding event; the method is thus suited to throughput screening to search for small molecules for drug development. Because the signal is based upon mass change at the surface, the study of molecular interactions using SPR

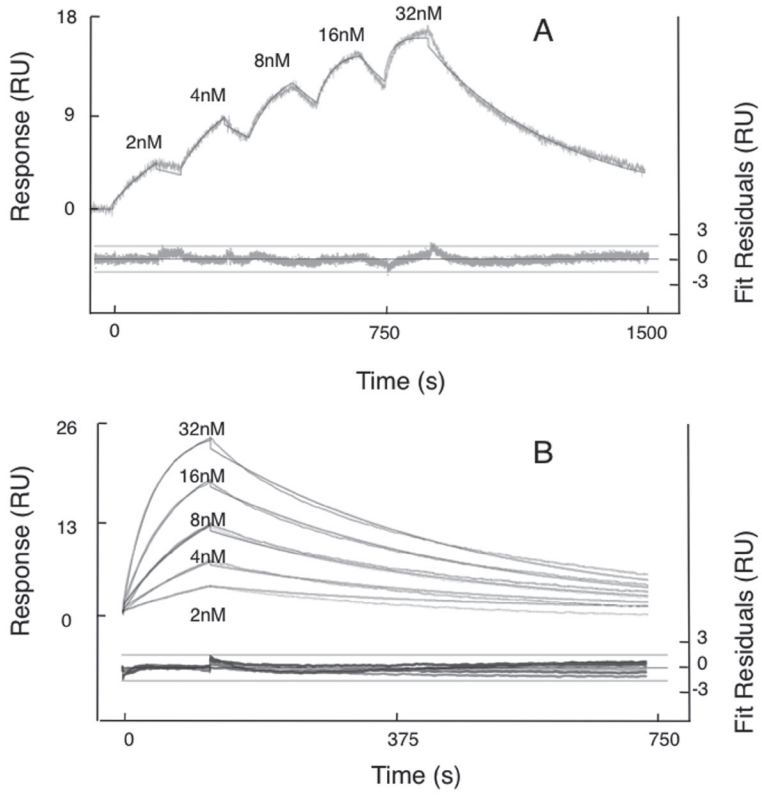

Figure 3. Single (A) and multi-cycle (B) kinetic analysis characterising the binding between beta-2-microglobulin by a monoclonal anti-beta-2-microglobulin antibody. Approximately 1400 RU of monoclonal anti-beta-2-microglobulin antibody was immobilised to a CM5 BIAcore® T200 sensor chip by amine coupling. The chip was washed with ethanolamine and then beta-2-microglobulin (2-32 nM) was injected at 30 microlitres/min over the sensor chip in a 10 mM Hepes, pH 7.4 running buffer containing 150 mM sodium chloride, 3 mM ethylenediaminetetraacetic acid and 0.05% P20 surfactant. Regeneration of the sensor chip was achieved using a 30 s injection of 10 mM glycine hydrochloride, pH 2.5 at a flow rate of 30 mL/min. The data were fitted with a 1:1 binding model. The fit residuals are shown below each fitted plot. For the multi-cycle kinetics (B), each successive run is overlayed ($Chi^2 = 0.205 RU^2$; $k_a = 0.7 \times 10^6\ M^{-1}s^{-1}$; $k_d = 0.003\ s^{-1}$; $K_D = 4.0 \times 10^{-9}$ M), whereas the one continuous run is shown for the single cycle kinetics (A) ($Chi^2 = 0.149\ RU^2$; $k_a = 1.4 \times 10^6\ M^{-1}s^{-1}$; $k_d = 0.003\ s^{-1}$; $K_D = 1.9 \times 10^{-9}$ M).

also enables, under particular conditions, calibration-free concentration analysis. That is, the concentration of analytes can be determined without a need for a calibrator, a highly useful feature discussed in more detail later in this review.

Limitations of SPR

One potential limitation of the SPR method is that the ligand may not maintain its native configuration upon immobilisation on the sensor chip surface, alternatively, its orientation may sterically hinder analyte binding. If required, the reverse or "upside down" orientation should be performed (the ligand becomes the analyte and *vice versa*). If this is not possible, then the ligand can be immobilised in a different way to address the effect of immobilisation on binding. Immobilisation strategies using antibodies, biotin or other "tags" that uniformly orient the ligand, may help avoid heterogeneity of binding due to different orientations of the ligand immobilised to the sensor surface. Another fundamental problem introduced when a ligand is physically immobilised on a surface is that it is physically separated from the analyte in the bulk solution. It is crucial to ensure that the transfer of the analyte to the ligand at the sensor surface is not limited, otherwise the analyte concentration near the surface will be different from the bulk concentration (referred to as mass transport limitation). Non-specific binding effects at the sensor surface must also be carefully controlled and this requires meticulous experimental design. The presence of low-affinity, nonspecific surface sites can also influence mass transfer.[14] Unfortunately, the history of SPR kinetic analysis is littered with poorly designed and controlled studies and invalid assumptions that lead to misinterpretation of data.[14,15] Accurate and appropriately interpreted kinetic data demands

a thorough understanding of binding kinetics and the potential sources of artefact. Rich and Myszka publish an annual review of papers in SPR, where they discuss the evolution of the technology and provide examples of how to (and not to) publish biosensor data.[15]

SPR Applications in Diagnostics

Given the diversity of targets and applications that can be studied by SPR (Table 2), it is perhaps not surprising that over 4000 journal articles have already been published using SPR as the detection method (Figure 1). However relatively few of these studies have examined analytes of relevance to laboratory medicine or of relevance to diagnostic samples such as serum, plasma, urine or saliva. While the number of examples that are relevant to clinical chemistry and laboratory medicine is beginning to grow, studies are largely at the proof-of-concept phase, and few have been compared in any rigorous fashion to contemporary methods in clinical use. However it is important to enumerate a number to highlight the potential scope. Firstly, a number of cardiac markers, including cardiac troponin-T,[16] myoglobin,[17] B-type natriuretic peptide,[18] C-reactive protein[19,20] and cardiac troponin-I,[21] have been measured in serum by SPR. Human thyroid stimulating hormone and growth hormone, follicle stimulating hormone and luteinising hormone have also been measured in serum and urine samples using a portable surface plasmon resonance (SPR) immunosensor.[22] Mean intra- and inter-day coefficients-of-variation were less than 7%, while batch-assay variability using different sensor surfaces was less than 5% for each analyte measured and each sensor chip could be used for over 100 consecutive assay cycles. The authors of this study concluded that SPR immunodetection of pituitary hormones in body fluid samples was a highly reliable tool for endocrine monitoring in laboratory and point-of-care settings (see POCT section below).[22]

Immunosensing SPR is also being actively developed to enable the detection of a number of tumour markers for cancer diagnosis. For example, prostate specific antigen (PSA)-alpha-1-antichymotrypsin complexes were measured in serum using SPR.[23] Ultralow concentration of PSA (10 fg/mL, ~ 300 atomole) has also been measured by SPR in sera using antibody bioconjugates on superparamagnetic particles for off-line antigen capture.[24] Similar methodology enabled the detection of ng/mL brain natiuretic peptide in plasma[25] and 100 pg/mL staphylococcal enterotoxin B in faeces.[26] In other applications, pg/mL quantities of interleukin-8 were detected in saliva by SPR to clearly differentiate healthy patients from those with oral cancer,[27] and human chorionic gonadotropin and activated leukocyte cell adhesion molecule were measured as tumour biomarkers in 10% blood plasma using a SPR sensor array that enabled high throughput analysis.[28] Together, these studies demonstrate that high throughput, rapid and real-time biosensing of low-abundance biomarkers by SPR in biological fluids is entirely feasible.

The SPR signal is directly proportional to molecular mass changes at the sensor surface and, all being ideal, molecules as small as 100 Da can be observed in real-time, as they bind to their target.[29] Thus, small molecule SPR applications of clinical relevance are rapidly growing. For example, cortisol and cortisone have been measured by SPR in saliva and urine samples.[30] The immunosensor used was highly specific with no significant interference observed with other closely related steroids (even when there was cross-reactivity of the immunosensor used, this would be apparent from the binding kinetics). The method also showed good correlation to liquid chromatography/tandem mass spectrometry methods. In another example, 3-nitrotyrosine in human urine was measured by SPR as an *in vivo* marker of oxidative damage.[31] Intra-assay and inter-assay variation coefficients were 5.5 and 7.7%, respectively, and a stable signal was observed from the one sensor chip, which was used for over 200 measurement cycles and 15 days of continuous operation. Other small molecule applications using SPR include quantitation of drugs such as morphine,[32] methamphetamine,[33] theophylline,[34] clenbuterol[35] and choramphenicol.[36] There is also rapidly increasing interest in developing SPR for the rapid detection and identification of snake toxins, paralytic shellfish toxins[37] and various neurotoxins, such as tetrodotoxin,[38] which is the neurotoxin found in toads and pufferfish.

A particularly useful feature of real-time biosensing by SPR is that it enables the active, nonprotein-bound concentration of an analyte in plasma to be directly determined. This feature is of particular utility to medical scientists, clinicians and the pharmaceutical industry because an analyte's efficacy is greatly influenced by the degree to which it binds to proteins in the blood or other fluids. A case example is warfarin, the most widely prescribed oral anticoagulant for the management of a number of thromboembolic disorders. The majority of warfarin is bound to plasma proteins,[39] but the concentration of free active warfarin can change rapidly depending on a wide range of factors including the presence of other drugs co-administered whilst on warfarin therapy.[40,41] This is concerning especially considering the narrow therapeutic index within which warfarin can be safely administered to patients.[39] Unfortunately there is little consistency among analytical methods for warfarin measurement, which rely on mathematical methods to obtain a measure of the patients international normalized ratio (INR).[39,40] Measurement of the unbound active warfarin would be especially useful in patients with fluctuating INR estimates that make their therapy very difficult to manage.[39] Clinical levels of active,

protein unbound warfarin have been measured directly by SPR using an inhibition immunoassay.[42] This method entails passing a mixture of plasma ultrafiltrate pre-equilibrated with a monoclonal antibody to warfarin over the surface of a sensor chip to which a warfarin derivative has been immobilised. A decrease in antibody binding to the sensor chip reflects antibody binding to non-protein bound warfarin in the plasma ultrafiltrate. The method is reproducible and robust for more than 1000 regeneration cycles, making the method cost-effective and amenable to throughput. Moreover, there is excellent correlation between this SPR method and an existing high-performance liquid chromatographic technique for the determination of protein-unbound warfarin in plasma ultrafiltrate.[42] Studies are now needed to determine whether this method provides more meaningful results than the INR currently in use. The direct measurement of the active unbound plasma levels of drugs by SPR may also be beneficial in neonates and children, where drug availability can be so changeable compared to adults[43] and in patients with renal dysfunction, who suffer a higher incidence of adverse drug reactions due to an impaired drug binding capacity of their albumin.[44]

The versatility of surface plasmon resonance extends beyond drug and protein-protein interactions, to the detection of gene sequences and aberrations.[45] Detection of single nucleotide polymorphisms by SPR has been demonstrated for both amplified and real genomic samples.[46-51] Surface plasmon resonance imaging (SPRI), in particular, has been used in a multi-array format for multiplex SNP analysis of genomic samples with the ability to delineate the degree of heterozygosity at a polymorphic site.[47] Although the sensitivity of SPR/SPRI in detecting DNA sequences and polymorphisms is dependent on the sequence, sample and technique, the detection of 41 zM of unamplified genomic DNA has been reported.[49] The diversity of SPR techniques used for the detection of SNP/genomic gene sequences is increasing with sequence specificity determined by nucleic/peptide nucleic acid probes and DNA mismatch, polymerase and restriction enzymes in direct ligand-analyte and sandwich-type formats.[46-50] Interestingly, an increasing number of researchers are using SPR not only to detect genetic polymorphisms but to assess the impact of these sequences where they occur in regulatory or coding gene regions on transcription rates and regulation.[51]

Application of SPR to detect DNA-DNA interactions has also been extended to rapidly and accurately identify mixed aerobic-anaerobic infections, providing a reliable alternative to bacterial culture for rapid bacteria detection.[52] In this study, SPR provided the same detection rate as the traditional culture method and could be completed within 15 minutes with low cost given the excellent stability and regeneration capacity of the sensor surface. Other microbiological applications of SPR have also been reported. For example, a SPR-based sandwich immunoassay for serotyping Salmonella was shown to be highly quantitative and not prone to the false-positives which can confound standard slide agglutination tests for bacterial pathogens.[53] Surface plasmon resonance offers not only sensitivity and specificity in the detection of gene sequences and aberrations, but represents a versatile, regenerable and reproducible technique amenable to clinical diagnostics.

Calibration Free Concentration Analysis (CFCA)

CFCA of an analyte under study is a powerful but unappreciated application of the SPR approach. Clearly any test that needs a standard or calibrant will only be as accurate as the standard or calibrant used, variations of which can arise from a multitude of factors. Furthermore, in many instances a protein or other standard may not be available. CFCA enables the concentration of the analyte to be determined without any need for a standard.[54] Thus CFCA has important ramifications for the disciplines of clinical chemistry and laboratory medicine as they move towards global standardisation through the implementation of metrologically correct measurement systems that can be reproduced from laboratory to laboratory.

Another major advantage of CFCA is that it gives the actual "active" concentration of an analyte. Because the method relies on the diffusion and actual binding of the analyte of interest to a ligand on the surface of a sensor chip, only active binding analyte is measured by CFCA. ELISA, spectroscopic and other methods that derive estimates of concentration against a known standard only provide an estimate of the total analyte. These methods are not able to distinguish between active and inactive analyte. Thus CFCA is likely to have a profound influence in pharmaceutical and other industries where the actual concentration of an active analyte (eg protein) is central to optimising production processes.

Presently the CFCA capability is offered only in the software associated with the Biacore X100, T100 and T200 instruments, but is likely to be quickly and broadly embraced across the SPR sector. The CFCA method does, however, have some limitations. It is only able to provide active concentration estimates for analytes that associate rapidly with their ligand (k_a >5 x 10^4 M^{-1} s^{-1}) and with some reasonable affinity (K_D <10^{-7} M). The method currently also assumes a 1:1 reversible binding stoichiometry, which may not be appropriate to every scenario. The diffusion coefficient of the analyte also must be known, but this presents less of a problem, as databases of diffusion coefficients for various analytes are available. If this inputted value of the diffusion constant differs from its true

value, then this will translate to an error in the concentration of the analyte determined by CFCA, by a factor raised to the power of two thirds (thus, underestimating the value of the diffusion coefficient of an analyte by two-fold, will lead to a concentration that is too high by a factor of about 1.6). Other factors such as the viscosity and temperature have small effects on the diffusion coefficient but are likely to be less significant that the curve fitting to data used in the CFCA. Nevertheless, provided different laboratories use a given diffusion coefficient for a given analyte, then the active concentration determined for any sample will be effectively standardised without the need for a standard or calibrant.

In brief, CFCA entails measuring the rate of binding of the analyte under conditions showing mass transport limitation. Two flow rates are required for reliable evaluation. Mass transport limitation occurs when the analyte associates (binds) or dissociates from the sensor surface quicker than it can diffuse in solution to and from the surface. Slow flow rate and large ligand densities will facilitate mass transport limitation. If there is no mass transport, the kinetic rate constants calculated will be independent of flow rates. This is preferred for kinetic experiments where the data must not be limited by mass transport, otherwise one simply measures the rate of diffusion rather than the actual interaction rate(s). Further details on CFCA theory can be found elsewhere.[54-56]

The software accompanying the Biacore X100, T100 and T200 instruments have an inbuilt wizard to guide the CFCA. The software performs evaluation using a 1:1 model, fitting for analyte concentration, where the molecular mass and the mass transport coefficient of the sample are known. As the initial binding rate is measured in this technique, the molecular mass and the mass transport coefficient (calculated from the flow rate, flow dimensions and diffusion coefficient) are provided by the user and the software is then capable of resolving the active concentration of the analyte. A recent article has demonstrated the applicability of CFCA for the quantification of monovalent vaccines.[57] With increasing population of SPR users, CFCA could potentially become a preferred method for the quantification of proteins and other biomolecules.

SPR, Lab-on-a-Chip (LOC) and Point-of-Care Testing (POCT*)*

"Biochip" and "lab-on-a-chip" technologies have grown rapidly over the last few years and this has coincided with increasing interest in POCT platforms (Figure 1).[58-60] Indeed, the investment in LOC and POCT technologies is aggressive and highlighted by the registration of over 200 microfluidics, lab-on-a-chip, BioMEMS (bio-micro-electro-mechanical-system) companies worldwide in 2011.[61]

SPR technology has many traits that make it ideal for the POCT and other field applications. It enables quantitative, label-free and real-time sensing of a wide range of clinically relevant targets within seconds and with very high levels of sensitivity and specificity. However, in the context of a POCT configuration, field applications of SPR have been generally hindered by the bulky nature of the detection apparatus. Considerable effort is now being expended to miniaturise SPR detection devices.[59,62] The Spreeta™ (Sensata Technologies Ltd) is a miniaturised SPR device that measures just 1.5×0.7×0.3cm that incorporates the optical system inside the sensor.[63] The device is claimed to have detection limits comparable to performance of a standard ELISA immunoassay. Biosurfit is developing Spinit, a proprietary technological platform for the POCT market, and they claim that their device is a user-friendly reader that will use one small drop of blood and gives precise results within 15 min for multi-parameter test panels.[64] Other hand-held devices have been described[65-69] and considerable further research is being undertaken to miniaturise these SPR detection platforms.[62,70]

Future Trends and Technology Perspectives

The demand for multiplexing of reactions and the need for sophisticated SPR microarrays is growing. For example, human chorionic gonadotropin and human albumin were measured on the same sensor surface in healthy human urine in a study of abortion and preterm delivery during early pregnancy.[71] In a quite different application, seven competitive immunoassays were multiplexed on a single sensor surface to develop a high throughput screening method to monitor antimicrobial drug residues at parts per billion in milk.[72] Biosensor chips and instruments are already available that include multiple channels in each chip and in array-based formats. For example, Biorad's ProteOn™ XPR36 instrument enables throughput using 6 x 6 array format that allows simultaneously analysis of 36 different protein interactions in real-time. The BiacoreTM4000 uses a 4 x 5 array and robotics that enables 4800 interactions to be analysed on 16 targets within 24 hours with exquisitely high levels of sensitivity.

SPR imaging (SPRi) is at the forefront of the technology. It uses a charge-coupled device camera that monitors binding events by recording local refractive index changes across the surface of a sensor chip.[73] This technology enables high level multiplexing that is suited to the type of high throughput demanded for proteomic investigations. A parallel microfluidic SPRi array enabled the binding events of up to 264 individually addressable chambers (for the attachment of up to 264 different ligands) to be interrogated against up to six analytes in a single experiment.[74] This array was used to study the antibody binding to anti-human alpha-thrombin and samples could be recovered without cross-contamination. In addition to these advanced microfluidic-

Table 3. Advantages and disadvantages of using SPR to monitor molecular interactions.

Advantages	Disadvantages
✓ Label-free enviroment	× Immobilisation effects
✓ Real-time, continuous measurement	× Steric hindrance with binding events
✓ Generic methods for diverse molecule sets	× Non-specific binding to surfaces
✓ Quick testing	× Mass transport limitations
✓ Small sample amounts & volumes	× Control experiments must be meticulously designed
✓ Highly sensitive	× Misinterpretation of data common
✓ Specific to the binding event	× Expense of sensor chips and instrumentation
✓ Sensor chips can be regenerated	
✓ Measure "active" concentrations	
✓ Concentration determined in absence of a standard (CFCA)	

based SPRi arrays, contact printing or pin-spotting of sensor surfaces can be used to produce high density arrays. For example, a 792-feature antibody microarray was fabricated using standard pin-spotting on bare gold substrates to enable comparison of the serum protein profiles from liver and non-liver cancer patients,[75] with, as might be predicted, an increase in alpha-fetoprotein being amongst 39 significant serum protein changes that were observed between the two groups. This study is one of many which shows that high throughput microarray systems can provide accurate and quantitative, label-free and real-time proteomics information in a manner that is fast and convenient. Plexera® Biosciences plans to launch a commercially available 1350-feature SPRi microarray targeting proteomic applications such as antibody library screening and biomarker panel profiling.[76]

Other SPR developments, such as localised surface plasmon resonance (LSPR), which utilises noble metal nanoparticles to enhance sensitivity, are also advancing and enable multiplexing.[77,78] Surface Enhanced Raman Scattering (SERS) has the potential to be combined with SPR to massively increase (10^{16}-10^{18}) the local electromagnetic field intensity of nanoparticles to a level far exceeding the single-molecule SERS detection limit[79] but to this point in time, this method lacks versatility.

Another important area in SPR research is in the design of the sensor chip interface where the biological interaction(s) occur and are probed. Novel sensor interfaces are being optimised to measure lectin-carbohydrate and lipid-protein interactions.[73] A nucleic acid aptamer-based biosensor surface has been developed for the detection of retinol binding protein 4 for the early diagnosis of type 2 diabetes.[80] This SPR biosensor produced better dose-dependent responses and was more sensitive than the ELISA assays.

Conclusion

The continued development of SPR, imaging systems, high throughput arrays and sensor interfaces and surfaces are continuing at a rapid pace and will make important contributions to our understanding of the complexity and dynamic nature of biological systems. SPR as a molecular profiling technology that enables proteomic, metabolomic and other molecular analyses to be undertaken in a real-time and label-free format will also impact on what is possible in future with LOC, POCT and personalised medicine, in particular.

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