

# Optical Biosensors: Making Sense of Interactions

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## Introduction

Biosensors have been described as the offspring of an arranged marriage between biology and electronics. The first commercially successful biosensors were developed in the 1960s as enzyme-based devices primarily for medical diagnostics. Currently, the fastest growth area involves affinity-based, label-free biosensors that yield real-time information on biomolecular interactions, as well as analyte concentrations in food, agricultural, nutraceutical, or environmental samples.

As illustrated schematically in Fig. 1, an affinity biosensor is essentially comprised of two distinct components: a biological recognition element, *e.g.* antibody, enzyme, lectin, receptor, nucleic acid or microbial cell, and (in close contact) a signal transducer, *e.g.* optical, acoustic, piezoelectric or electrochemical, connected to a detector for data acquisition and processing. The signal from the interaction of analyte with the biological element is converted to a quantifiable signal.

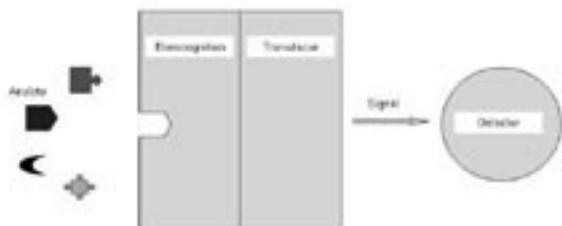


Fig. 1. Schematic of a biosensor.

Several transducer techniques are used in biosensor devices as indicated in Fig. 2. The most commonly reported optical transducer techniques include the variants fibre optic, surface plasmon resonance (SPR), optical grating coupler, *e.g.* optical waveguide light mode spectroscopy, and evanescent wave. Amongst these the SPR-based biosensors are currently the most commonly applied.

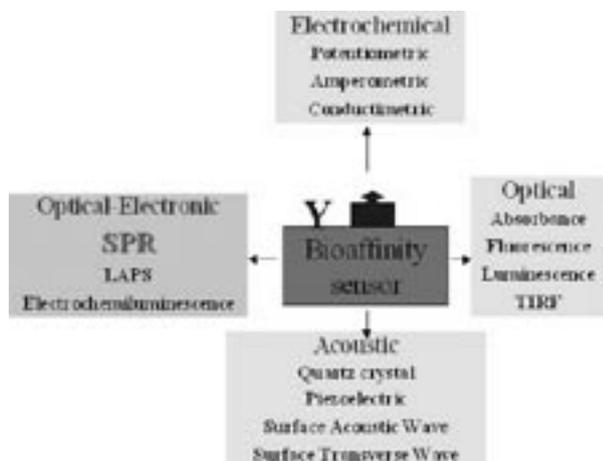


Fig. 2. Overview of transducer alternatives for affinity-based biosensors.

An SPR system generally requires a laser light source, detector, glass prism, and gold surface, (Fig. 3). In an angle-shift instrument, total internal reflection of incident polarised light is configured at the interface of the gold-covered glass and a continuously flowing liquid medium. The electromagnetic field component penetrates the metal and transfers some energy to the valence electrons thereby producing charge density waves known as *surface plasmons* at the interface. The resonance conditions that produce a reflected light intensity minimum are influenced by changes in refractive index at the interface. Thus, any mass changes due to association and dissociation events with immobilised probes at the sensor surface are monitored in the form of a continuous plot of refractive index against time. The significant advantages inherent to such systems are that the transduced signal is acquired both in real-time and label-free.

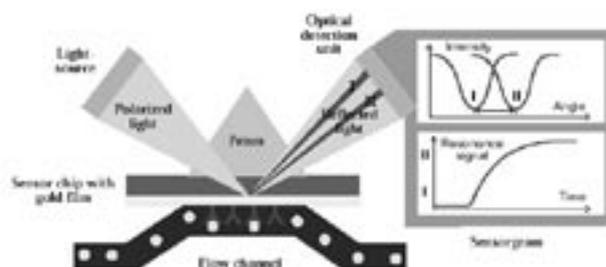


Fig. 3. Schematic of integration of Surface Plasmon Resonance (SPR) optics, sensor surface, and flowcell in a Biacore instrument, reproduced with permission from Biacore®.

The continuous output is in the form of a sensorgram that follows the association and dissociation events within the evanescent field between analyte in solution and the immobilised ligand on the sensor surface. In a typical schematic sensorgram (Fig. 4) the SPR response [in resonance units (RU)] is monitored against time. Unless dissociation is very rapid, a regeneration step is needed to re-establish a fully functional immobilised surface for subsequent cycles.

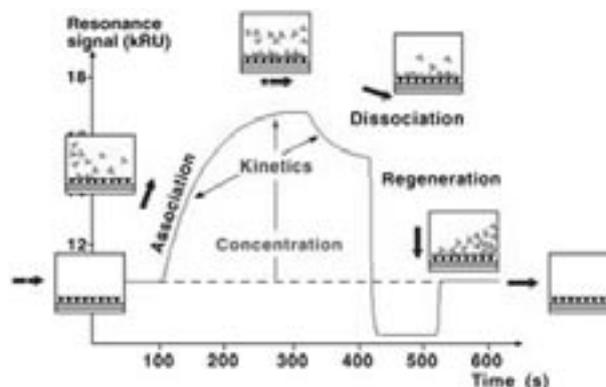


Fig. 4. Schematic SPR-generated sensorgram, illustrating both events and extractable information over time, reproduced with permission from Biacore®.

The information-rich sensorgram, dependent upon the experimental conditions under which it is generated, can yield data related to:

- *Specificity* – the extent to which different molecules interact with a single partner immobilized on a sensor surface.
- *Kinetics* – the rates of a biological interaction, both of complex formation ( $k_a$ ) and dissociation ( $k_d$ ).
- *Affinity* – the binding strength ( $K_D$ ) that can be determined either from the level of binding at equilibrium as a function of sample concentration, or from the ratio of the kinetic rate constants ( $k_d/k_a$ ) under defined conditions.
- *Concentration* – determined by monitoring the interaction of either a pure analyte or one in a complex mixture, e.g. serum and food samples, over a prepared sensor surface. Direct, sandwich, or indirect assay formats may be used, with quantitation of the target analyte in unknown sample achieved by interpolation of binding response on a calibration curve acquired with authentic standards. Unlike physicochemical separation techniques, SPR-based immunoassay provides an estimate of the biologically active analyte content, in contrast to total concentration, a feature that is particularly significant when structurally labile proteins are of interest.

While there are now several commercial vendors of pre-configured *closed-box* SPR-based instruments, e.g. Biacore, Affinity Sensors, IBIS Technologies, and Texas Instruments, that includes the growing diversification into imaging technologies, Biacore® is globally dominant for the conventional SPR applications described above. Since the first commercial SPR-based system released in 1990, there has been a significant growth in publications (mostly in life science and drug discovery applications) with a total of ca. 4,500 articles to the end of 2004. Excellent reviews of affinity biosensors and SPR are available.<sup>1-3</sup>

### What's Happening in New Zealand?

There are currently eight Biacore instruments here. Five are designed for the development and routine use of concentration immunoassays in foods and other biological materials, and three are configured for higher resolution kinetics and affinity applications. In comparison, Australia has 45-50 instruments with major applications to life science in academia, and to biotechnology that focuses on antibody therapeutics, drug discovery, membrane interactions, DNA interactions, and receptor-ligand and protein-protein interactions.

Analytes relevant to the food industry can generally be assigned among three categories when targeted for concentration analysis. The first includes low molecular weight nutrients or contaminants that are usually determined by chromatographic methods. The second group includes various proteins or other immunogenic macromolecules for which conventional immunoassay techniques are used, while the last group includes intact microorganisms, generally assayed by microbiological methods. Biosensor techniques based on SPR-based concentration immuno-

assay of food components can be configured for each of these analyte categories and there are now ca. 100 peer-reviewed publications describing specific applications to vitamins, pathogenic microorganisms,  $\beta$ -lactam antibiotics, steroid hormones, proteins, food adulteration, allergens, drug residues, marine biotoxins, coumarins, and GMOs. In fact, publications related to food analysis are one of the fastest growing applications of SPR-based systems.

In general, the sensitivity, specificity, and quantification range of bio-specific methods are determined by ligand-analyte affinity. Therefore, an optimized SPR-immunoassay relies predominantly on appropriate ligand selection and immobilisation chemistry, as well as buffer conditions, contact time, and the regeneration protocol.

## SPR and Food Analysis

### Vitamins

Within Fonterra, the principal usage of SPR instrumentation has been in the development and application of immunoassays for routine compliance testing of specific B-group vitamins in supplemented cow milk-based paediatric formulae, and other milk-based foods.

B-group vitamins are commonly consumed as dietary supplements to the extent that ca. 60% of respondents in a survey reported their use, although they are now being challenged by new botanical ingredients. Nevertheless, vitamins are produced commercially to more than 100,000 tons per annum, representing an almost \$7 billion industry in the US alone. The water-soluble vitamins folic acid, biotin, riboflavin, pantothenic acid, and vitamin B<sub>12</sub> are added as supplements to many foodstuffs and animal feeds. For infant formulae designed to substitute for breast milk, their inclusion is legally required. Thus, reliable and accurate concentration estimates of these vitamins are mandatory for compliance during food processing to ensure that labelling needs are met and that vitamin dosage is controlled during the production of fortified products. Furthermore, the demands of the modern processing environment and the sensitivity of vitamins to environmental factors demand a rapid analytical procedure.

Traditionally, the concentration of B-group vitamins is measured using microbiological assays that require a high level of skill, and suffer from relatively poor precision. HPLC-based methodologies are also available but reliable results from complex food matrices such as milk are difficult to reproduce and are not yet routine for biotin, folate and vitamin B<sub>12</sub>. These assays are especially challenging because of the very low levels present and the high lability of the vitamins during multiple sample extraction steps. SPR-immunoassays that yield vitamin concentrations utilising Biacore instrumentation are configured with an inhibition format, and have been demonstrated to be accurate, rapid, sensitive, and highly suited to a routine high-throughput compliance program. As with immunoassays in general, the biological specificity of the detecting molecule results in the need for minimal sample preparation. With a single instrument and a range of vitamin-specific kits, a laboratory is equipped to fulfil the requirements of food and dietary supplement manufacturers, international

regulatory bodies, and consumers.<sup>4-7</sup>

AgriQuality Laboratory Services (Auckland) uses a Biacore Q for a range of compliance concentration assays for folic acid, biotin, and vitamin B<sub>12</sub> in fortified dairy products and foods. They are also evaluating the technique for the routine detection of antibiotic residues. The assays are kit-based with standards, antibody/antigen, sensor chip, and running buffers included. In general, vitamin supplements in foods present no particular difficulties except when starch is present, *e.g.* in cereal foods. For such cases, and when endogenous vitamins are required for analysis, more sophisticated sample preparation is necessary for analyte release prior to the SPR-based immunoassay. Thus, sample preparation protocols that are recommended with the kits have been modified to allow several food matrices to be tested to verify fortification levels. Current work is proceeding to optimise such strategies, incorporating various enzyme treatments, protein precipitation, and acid or alkaline hydrolysis for more reliable estimates of food folate.<sup>8</sup>

### **Milk Proteins**

Milk contains numerous minor proteins with physiological properties targeted at providing immunoprotective, growth, and antimicrobial factors to the neonate. These are distinct from the nutritionally more significant major proteins. Many of these minor bioactive proteins are found in the whey fraction of mammalian milks, and are generally present at elevated levels in colostrum, reflecting their importance to early neonatal health. The increasing commercial interest in exploiting their therapeutic value has stimulated the need for reliable concentration assays for their determination at naturally occurring levels in milk and colostrum, at supplemental levels in infant formulas, and at pharmaceutical levels in milk protein isolates and nutraceuticals. Both liquid chromatographic (reversed-phase, ion-exchange and affinity) and conventional immunological methods (immunodiffusion, nephelometry and ELISA - *Enzyme-Linked Immunosorbent Assay*) exist for these proteins. Despite this, Fonterra has successfully developed and applied SPR immunoassay techniques for immunoglobulin G (IgG), folate binding protein (FBP), lactoferrin (Lf), and lactoperoxidase (LPO) in bovine milk and colostrum.<sup>9</sup>

Sensor surfaces were prepared with affinity-purified goat anti-bovine IgG, folic acid derivative, goat anti-bovine Lf, or rabbit anti-LPO on a carboxymethyl-dextran-coated gold sensor chip (CM5) via amine coupling under instrument control. Samples were prepared for analysis by dilution to between 1:1,000 and 1:50,000 in an Hepes buffer, depending on analyte and level. Calibration curves were established by serial dilutions of authentic standards in buffer. Calibration standards and sample extracts were dispensed into 96-well microtitre plates and injected for 3 min. at 20  $\mu$ L/min (IgG), 8 min. at 20  $\mu$ L/min (FBP) or 5 min. at 10  $\mu$ L/min. (Lf and LPO). Binding responses acquired 30 sec. after the end of the injection were measured relative to the initial baseline and used for generation of the calibration curve and interpolation of unknown samples. The surface was regenerated by injection of 25 mM

phosphoric acid (IgG), 75 mM sodium hydroxide (FBP), 10 mM glycine-HCl, pH 1.75 (Lf), or 10 mM glycine-HCl, pH 1.50 (LPO).

The *on-farm* extraction of Lf from milk during milking has been achieved at Dexcel. This was the culmination of work undertaken over 18 months by a team involving dairy farmers, Dexcel, Waikato University, and Sorsotec. The automated on-line fractionation unit, named *Bruce*, extracted Lf via a single-stage stirred tank with cation exchangers SP Sepharose Big Beads™ while the cow was being milked in Dexcel's robotic milking unit.<sup>10</sup> Lf was measured using SPR technology with a Biacore 3000 and this allowed for the rapid measurements of yields from different sample matrices such as raw whole milk (feed), processed milk, and eluates.

The project was designed to prove that minor, high-value milk components could be extracted from whole milk with minimal disruption to the milking routine or to the bulk milk composition during milking.<sup>11</sup> In the initial run, *Bruce* continually extracted Lf from milk during the normal operation of the robotic milking process in which the cow presents herself for milking. The on-farm system proved effective and yielded 15% more Lf than is achieved in conventional factory processing systems. Similar work at Waikato University has targeted the optimisation of a rotating bed annular chromatographic system for the continuous separation of milk proteins including BSA and Lf.

### **Progesterone and Estrogen Steroids**

HortResearch in Hamilton have utilised SPR technology in developing a number of high sensitivity immunoassays for progesterone in milk, in view of the value of this steroid hormone as an indicator of bovine oestrous.<sup>12,13</sup> They utilised Biacore instrumentation, either as a final assay technique or for evaluation of antigen:antibody binding characteristics as a means to screen optimal binding partners for ELISA assay. Inhibition assay formats utilising monoclonal antibodies to progesterone have been exploited, with the progesterone immobilised directly via various length linkers, or via a protein conjugate. Strategies have included novel surface attachment and gold-tagged enhancement labels for binding antibody that have increased sensitivity and reduced detection limits by orders of magnitude. The surface chemistry enables >1000 cycles/chip with excellent precision, and the surface linkers facilitate projection of antigen into the fluid stream.

In addition to assay design and enhancement, SPR has been used to study the effects of conjugation chemistry on the ability of antibodies to recognize targets, which is very important for both biosensor construction and the design of small molecule-drug conjugates. Such studies include assessments of the effects of changing conjugation position on antibody binding for immobilised steroids such as estrogen, and developing new synthetic routes for producing derivatives with maximal antibody recognition properties.<sup>14</sup>

Sorsotec, in conjunction with the Waikato University, is currently involved in using SPR technology to measure

and/or confirm binding between progesterone and a conjugated enzyme-antibody for use in an alternative ELISA immunoassay system. The aim is to commercially exploit analysis of progesterone for improved on-farm monitoring of herd fertility.<sup>15</sup>

## SPR and Biotechnology

### PEGylated Proteins

The *in vivo* stability of proteins used for clinical and therapeutic use is vital. The Bioseparations and Biomolecular Engineering group at Waikato University (now located at Canterbury) has explored the interaction of PEGylated proteins with various surfaces using SPR. PEGylation is the covalent attachment of polyethylene glycol groups to the target drug and is a technique that has been reported to prolong the half-life of the therapeutic protein. Such surface interactions of biomaterials are fundamental to issues such as host response and biocompatibility, and SPR offers a valuable alternative to other techniques, such as quartz crystal microbalance, for their study.<sup>16</sup>

### Antibody Characterisation

HortResearch (Palmerston North) implemented SPR in 1997 and was the first NZ laboratory to do so. It has had much success since then, through use of a manual Biacore X instrument, in the development of antibody-based protein arrays. Host systems designed for multiple protein expression have been established for the production of monoclonal antibodies. The instrument is used for epitope mapping of the produced antibodies, to determine the affinity constants ( $K_D$ ) of the antibodies for their antigen, and for studying protein-protein interactions.

The Biacore X has also been used to develop competitive immunoassays for small molecules such as organophosphate pesticides, antibiotics, and phytotoxins. For such work, the group has developed their own monoclonal antibodies and synthetic haptens.<sup>17</sup>

### Microbial Pathogenesis

At Auckland University, the Molecular Bio-discovery team are investigating the mechanisms of microbial pathogenesis. A Biacore 2000 has been used to study the interaction between secreted pathogenicity factors from *Staphylococcus aureus* and *Streptococcus pyogenes* and components of the innate and adaptive immune response.<sup>18</sup> Common studies include the rapid screening of toxin mutants to identify and map binding sites that can then be combined with protein crystallographic studies to provide a complete picture of the *modus operandi* of individual toxins. Analysis involves qualitative comparisons of mutant binding with wild-type toxins to their targets coupled to sensor chips, followed by quantitative analyses to determine association ( $k_a$ ) and dissociation ( $k_d$ ) rates and the calculation of  $K_D$  to provide information on affinity.

One example of past studies is the analysis of the binding of the potent super antigenic toxin SMEZ-2 to soluble recombinant forms of Major Histocompatibility Complex Class II and T Cell Receptor molecules. SPR was employed to demonstrate that mutants of SMEZ had reduced

affinity to the T Cell Receptor in comparison to the wild type toxin. These results were used in conjunction with other immunoassays to confirm the exact location of the TcR binding site, and the effects of individual mutations in the binding site on the toxicity of SMEZ-2. The studies were essential in the construction of a modified toxoid version of SMEZ-2 that forms the basis of a commercial vaccine conjugate to enhance the immunogenicity of proteins, peptides and carbohydrate antigens.

This group has also studied the interaction between the human epithelial  $\text{Na}^+$  channel (hENaC) and domains of human ubiquitin-protein ligase hNedd4, where SPR was utilised to characterise the interaction affinities of synthetic binding domains of proteins.<sup>19</sup> Other studies have reported nanomolar estimates of  $K_D$  for the interaction between staphylococcal superantigen-like protein SSL-7 and IgA by both kinetic and equilibrium affinity experiments.<sup>20</sup>

### SPR-Mass Spectrometry

The ability to use SPR to both confirm the presence of a binding partner and extract its interaction kinetics and affinity is enhanced by the opportunity to identify structure via coupled mass spectrometry. A group at AgResearch have utilised the capability of the Biacore 3000 to assist identification of a binding partner to a newly identified protein associated with muscle atrophy and plan to utilise SPR-MS to structurally characterise the binding protein.

### Problems Inherent to SPR

Most of the difficulties with SPR measurements originate with the sample rather than the optics, which are relatively problem-free. Although ligand immobilisation is always required for an SPR experiment, there are a number of strategies that cover most instances. Non-specific interactions and bulk refractive index differences are probably the most difficult issues to deal with. It has been stated<sup>21</sup> that *the good news is that everything has an SPR signal and the bad news is that everything has an SPR signal* and hence, overcoming potential non-specific interactions represents the major challenge for both kinetic and concentration applications of SPR detection.

### Conclusions

In food compliance alone, the global food diagnostics market currently stands at *ca.* \$US 1.4 billion, and the application of biosensors is currently under-exploited, perhaps because of both an inherent conservatism and an occasionally inflexible regulatory system within this industry. The basic SPR instrument is not expected to change dramatically in the future, although there is potential for low-cost and field-type alternatives. Although acceptance of SPR technology has been relatively slow in NZ, recent installations have dramatically increased the visibility of this tool and it is likely that its attributes will become more apparent across analytical chemistry.

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