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APPLICATION BRIEF #004

Spreeta™

On the Sensitivity of Spreeta

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On the Sensitivity of Spreeta

ABSTRACT

A competitive assay format is demonstrated for the determination of biotin using Spreeta technology. In this assay biotinylated bovine serum albumin is physisorbed directly to the gold surface. Solutions containing a fixed concentration of antibody and variable concentrations of free biotin are premixed and analyzed.

SENSITIVITY AT THE GOLD SURFACE

Surface plasmon resonance is an optical technique that enables real-time monitoring of changes in the refractive index of a thin film close to the sensing surface. The evanescent field created at the surface decays exponentially from the surface and falls to one third of its maximum intensity at approximately 200 nm from the surface. With this in mind it is clear that the SPR technique is more sensitive at the gold surface than 200 nm from the gold surface and is almost completely insensitive to refractive index variations greater than 300 nm from the surface. This is a concern when large particles are bound to the surface. For example the bulk of a bacterial cell (1 μm in diameter) bound to the surface will remain outside the volume probed by the evanescent field. Even with maximum packing of the cells at the surface only a fraction of the volume probed by the evanescent field will be occupied by the cells. This restricts the binding responses that are attainable when large particles bind. Nevertheless, despite this limitation bacterial cells have been successfully detected as have red blood cells.
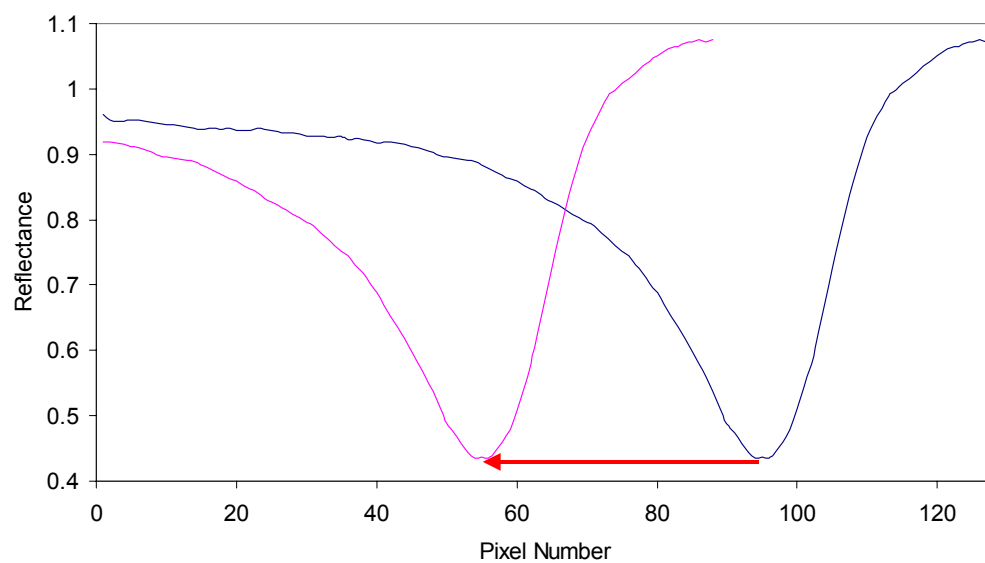
Figure 1. Overlaid SPR reflectance minima.
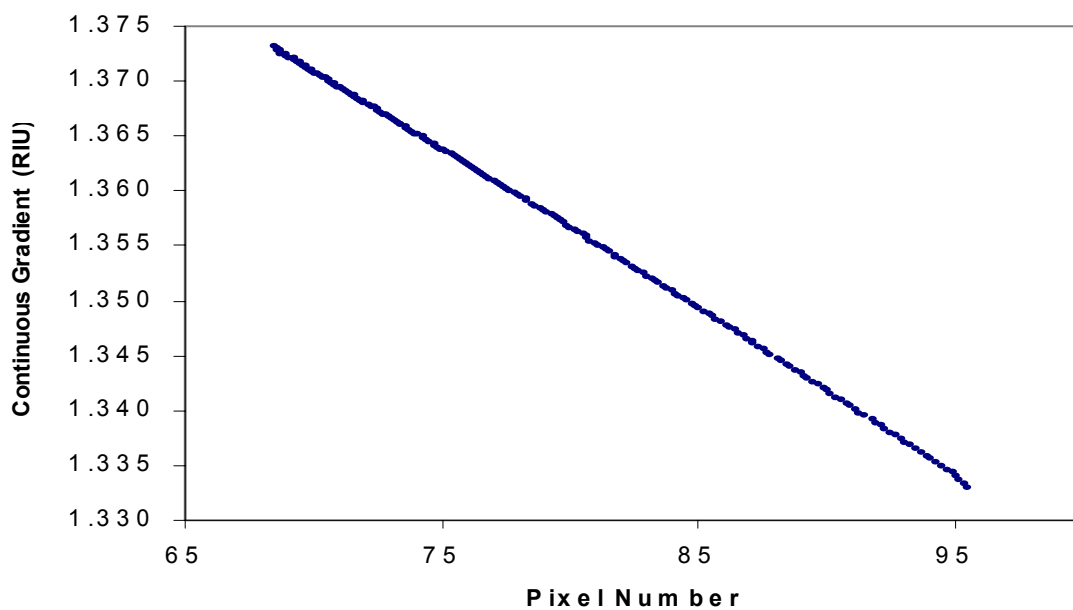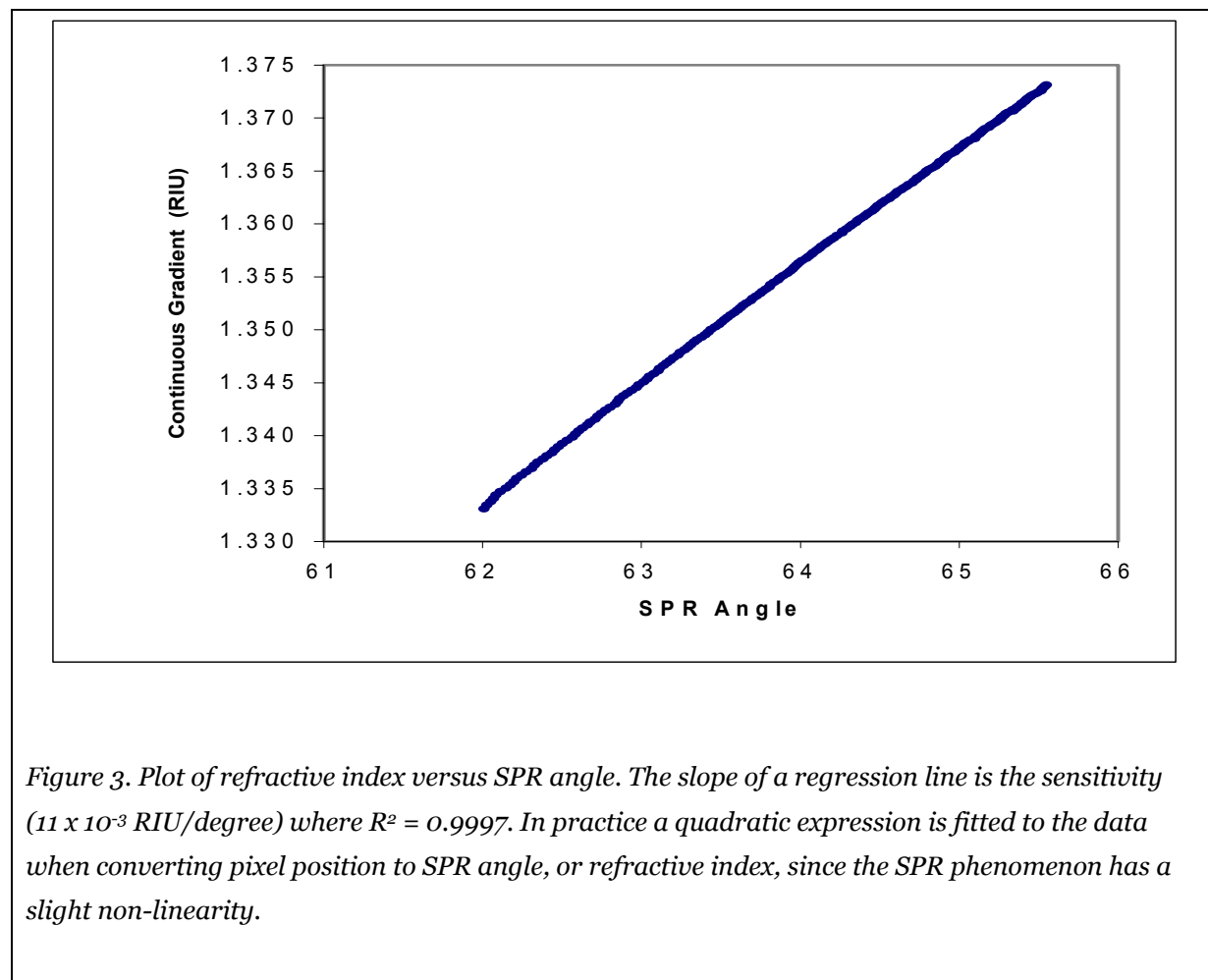
Transducer Sensitivity

Figure 2: Plot of the pixel position of the SPR minimum versus refractive index.

A minimum tracking algorithm is employed to continuously monitor the position of this minimum as it traverses along the photodiode array. This pixel position is then related to refractive index as shown in Figure 2 and may be used in calibrating the sensor. A solution of increasing refractive index was exposed to the sensing surface and the pixel position of the reflectance minimum was recorded. The slope of the plot (sensitivity profile) defines the sensitivity of the Spreeta sensor. However, it is more appropriate to define the sensitivity of the SPR transducer in terms of the SPR angle rather than pixel position. Obviously it is not possible to compare sensitivity values from one SPR transducer to another without doing this. Hence Figure 3 is the correct expression of the sensitivity of the Spreeta 2000 sensor.

Figure 3. Plot of refractive index versus SPR angle. The slope of a regression line is the sensitivity (11 x 10^{-3} RIU/degree) where R^2 = 0.9997. In practice a quadratic expression is fitted to the data when converting pixel position to SPR angle, or refractive index, since the SPR phenomenon has a slight non-linearity.
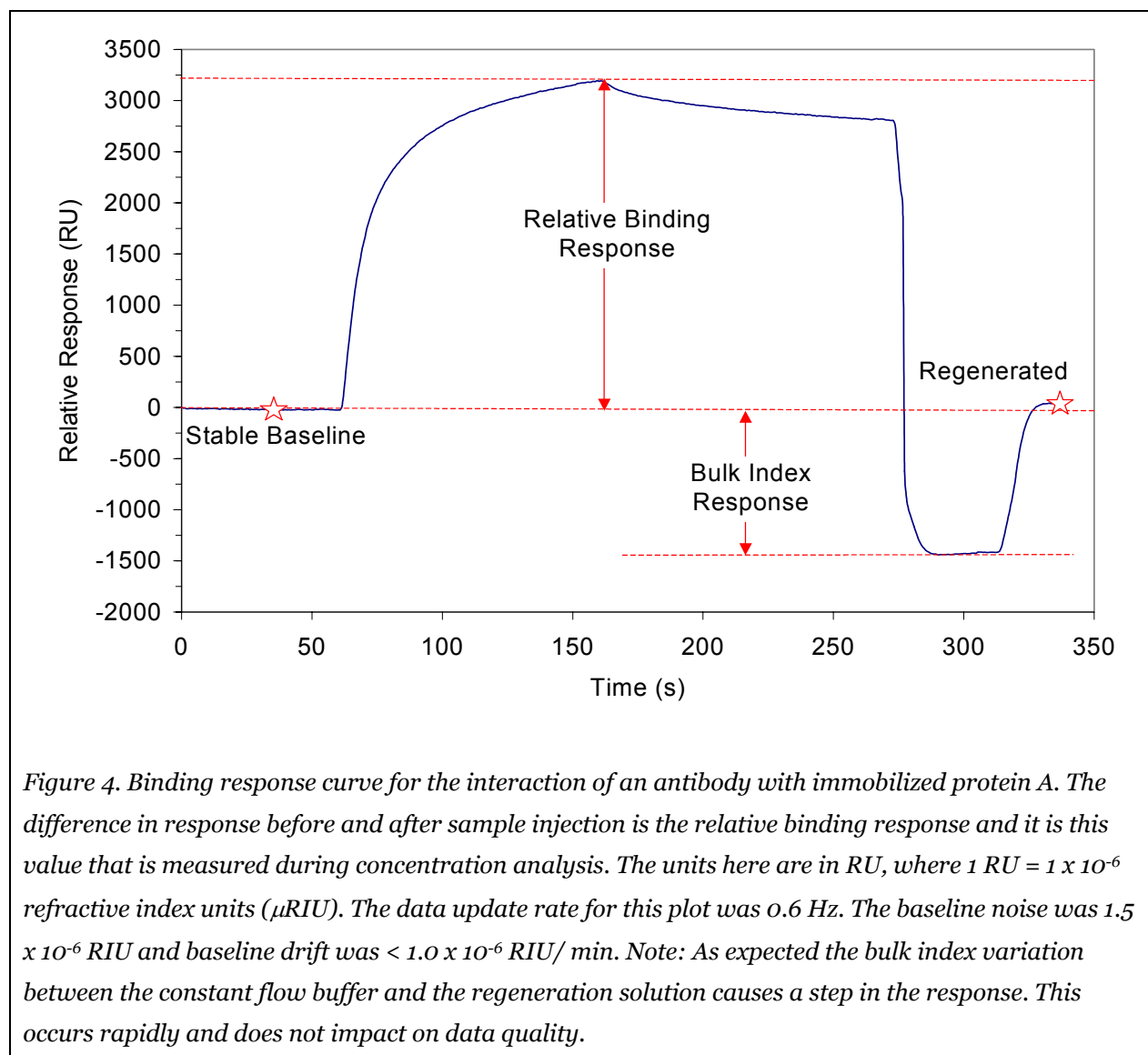
(It is important to note that any deviations in the shape of the SPR curves shown in Figure 1 will introduce distortions in the smooth sensitivity profile shown in Figure's 2 & 3. These can occur due to incorrect background referencing, debris on the surface or scratches in the gold etc.)

Limit of Detection

Many biochemists use the term sensitivity and limit of detection interchangeably. It is more correct to define sensitivity as stated above and use the term limit of detection independently. The fact that an SPR sensor doesn't directly measure mass but measures the refractive index change that results from a change in mass complicates the definition of the limit of detection.

In effect there are two correct values. As a surface sensitive refractometer the limit of detection for an SPR transducer may be defined as the minimal refractive index change that is detectable at three times the standard deviation of the signal noise (baseline noise). Obviously lowering the noise level by acquiring data faster and performing more averaging for each refractive index point will enhance performance.

Figure 4. Binding response curve for the interaction of an antibody with immobilized protein A. The difference in response before and after sample injection is the relative binding response and it is this value that is measured during concentration analysis. The units here are in RU, where 1 RU = 1 x 10^{-6} refractive index units (μRIU). The data update rate for this plot was 0.6 Hz. The baseline noise was 1.5 x 10^{-6} RIU and baseline drift was < 1.0 x 10^{-6} RIU/ min. Note: As expected the bulk index variation between the constant flow buffer and the regeneration solution causes a step in the response. This occurs rapidly and does not impact on data quality.

With optimized data acquisition (300 Hz) more averages may be taken thus reducing the noise level since the basline noise level scales according to $\sqrt{n}$, where n is the number of averages. For instance, if data is acquired at 180 Hz and one averages 100 of these SPR spectra per reading then the baseline noise decreases to ~ 0.3 x 10^{-6} RIU and the data update rate for the response plot will be 1.88 Hz. Hence the limit of detection will be 1 x 10^{-6} RIU. Temperature control is important as changes in temperature give rise to a drifting baseline. Obviously this must be controlled in order to make accurate measurements. A change of 1 °C will cause a baseline drift of 1 x 10^{-4} RIU if uncorrected. The system can be set up to self compensate for large temperature effects. Also thermostat control is a good option.

Analyte Limit of Detection

An application specific means of defining the limit of detection is to quote the minimal concentration of the target analyte that can be detected such that the response is 3 standard deviations above the uncertainty in the background response i.e. the analyte limit of detection. Hence if the limit of detection of the transducer is 1 x 10^{-6} RIU then the best limit of detection by definition will be 1 pg/mm^2, since 1 pg/mm^2 protein is equivalent to a protein mass change of 1 pg/mm^2. Ideally if one injected 1 pg of protein over a ligand coated SPR surface (area of 1 mm^2) and all the material bound then a response of 1 x 10^{-6} RIU would be obtained. However, there are many reasons why this is rarely possible and I discuss these later.

In Figure 5 a four parameter logistic expression is fitted to the data and the response from unknown samples is used to back calculate concentration values. The limit of detection is defined as the concentration that is readable by backcalculation that is 3 standard deviations greater than the background response. The background is the root mean square difference in the responses obtained for negative control samples interacting with the surface. There are many other ways of treating such data and the example presented is simplified.

(a) Overlaid binding interaction curves for 1 set of data over a range of mouse IgG concentrations. These are the raw interaction curves. Regeneration was accomplished using 20 mM HCl

(b) Titration curve for the interaction of mouse IgG with immobilized Protein A. The limit of detection is about 10 ng/ml.
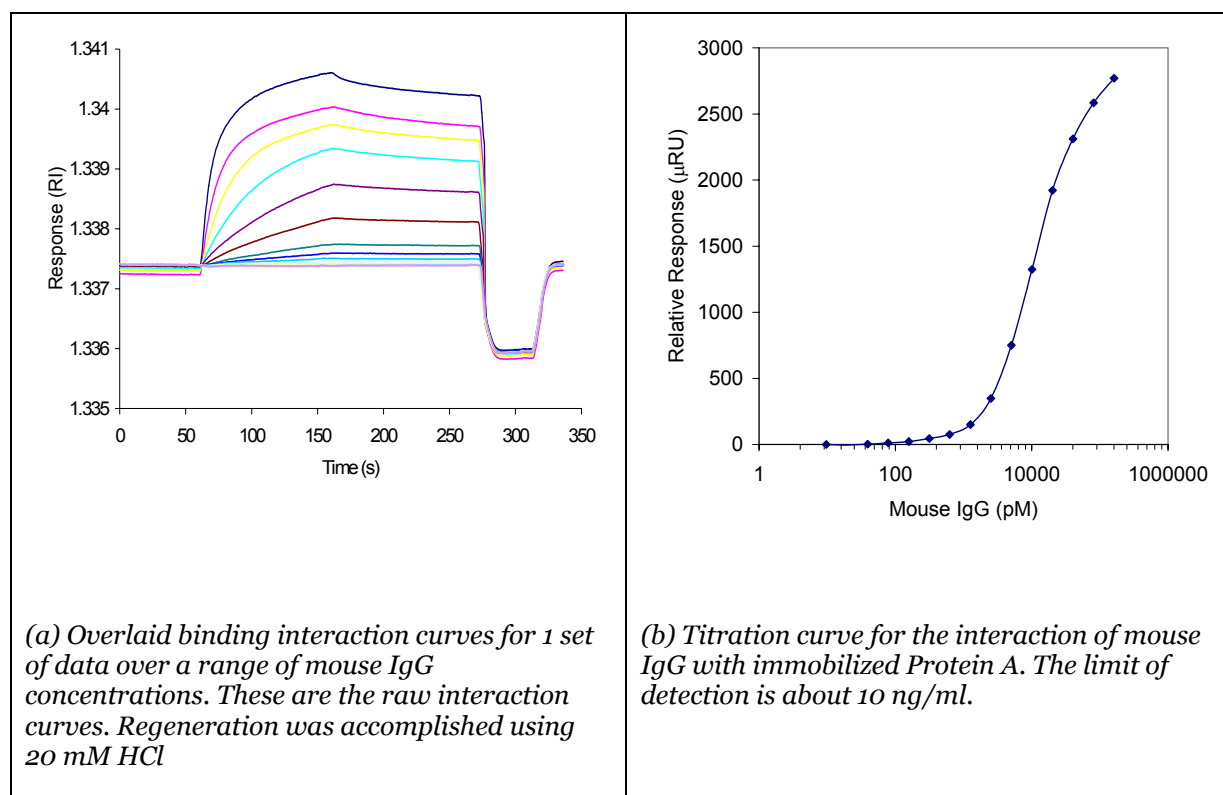
Figure 5. Assay for mouse IgG. The relative binding response, obtained as shown in figure 4, for a range of mouse IgG concentrations (see (a)) were plotted (see (b)). The sigmoidal curve defines the limits of the assay.

This appears very simple but there are a large number of factors that influence the analyte detection limit. **Simply put, the limit of detection and the measuring range as quoted in RIU defines the transducer efficiency. Given equivalent transducers then it is the design of the peripheral technology that dictates the overall performance of the system as a biosensor.**

SPREETA is currently available as a test kit and not an instrument. No effort has been made to optimize peripheral technologies. Values for the limit of detection (sometimes referred to as sensitivity) for SPREETA for a given analyte are misleading since these values are only applicable to the integrated system. **In fact the analyte limit of detection for the same assay conducted using a Spreeta transducer with differing peripheral technologies may vary 1000 fold or more.** Most of the literature concerning the sensitivity and limit of detection of SPR technologies neglect to mention these factors thus giving a false performance comparison.

The following is a brief account of the influence of peripheral technologies.

Flow Cell Design

The SPR signal is an averaged signal over the interaction area surface. Transport of analyte to the surface results from convective and diffusion forces. These phenomenon are described by the mass transfer coefficient (k_m) and are related to the flow cell design and operational flow rate by:

$$k_m = C_{lm} \cdot \sqrt[3]{\frac{D^2 . F}{h^2 . b . l_2}} \qquad (1)$$

where

D = Diffusion coefficient of the analyte(m^2/s)

$h, b \& l$ = Height, width and length of flow cell(m)

F = Bulk flow rate($\mu l/min$)

where

$$C_{lm} = 1.47 . \frac{1 - (l_1 / l_2)^{2/3}}{1 - l_1 / l_2} \qquad (2)$$

where

l_1 and l_2 are the start and end of the active sensing region relative to the inlet position

Stringent biomolecular interaction applications require that a stable analyte concentration gradient exists and this requires laminar flow conditions. Laminar flow conditions do not exist at the inlet or outlet and a time interval is required to develop full laminar flow. The time required for full laminar flow development assuming 100% MTL is given by the transient phase (TP) time and can be estimated from the expression:

$$TP = t . \sqrt[3]{\frac{36 . D . F^2}{h^4 . b^2 . l^2{}_2}} \qquad (3)$$

When TP is approximately 1.2 then laminar flow will predominate. Expression (3) shows the high dependency on the height of the flow cell (power of 4) and the square dependence on both the width and length. Clearly, all dimensions must be minimized to ensure optimum performance. We are restricted in our value for the width and length but may be able to reduce the height below 0.05 mm if we are to obtain high biosensing performance using a flow cell configuration. Figure 6 shows the time necessary for flow cells of various dimensions to establish laminar flow. Laminar flow conditions are required for the setup of stable concentration gradients and this is a prerequisite for real time biomolecular interaction analysis. It is possible to operate the biosensor under non-laminar flow condition but this is not optimal and will not enable us to utilize the full potential of Spreeta when using a flow cell.
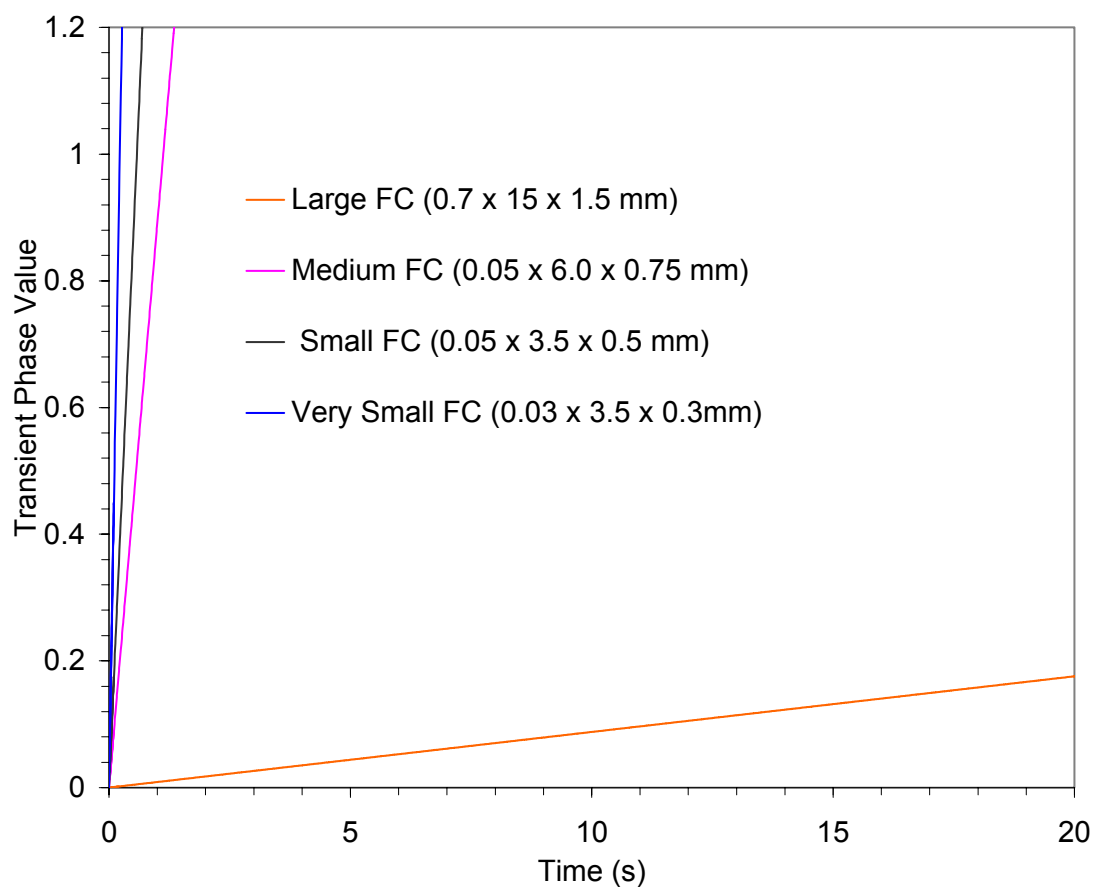
Figure 6. Laminar Flow predominates when the transient phase value reaches 1.2. It is clear that a small to medium flow cell is required to ensure laminar flow is establishing quickly and ensure stable gradients at the surface. For example the medium flow cell (FC) requires 1.5 seconds to establish laminar flow. It is not possible to establish laminar flow in reasonable time when using large flow cells.
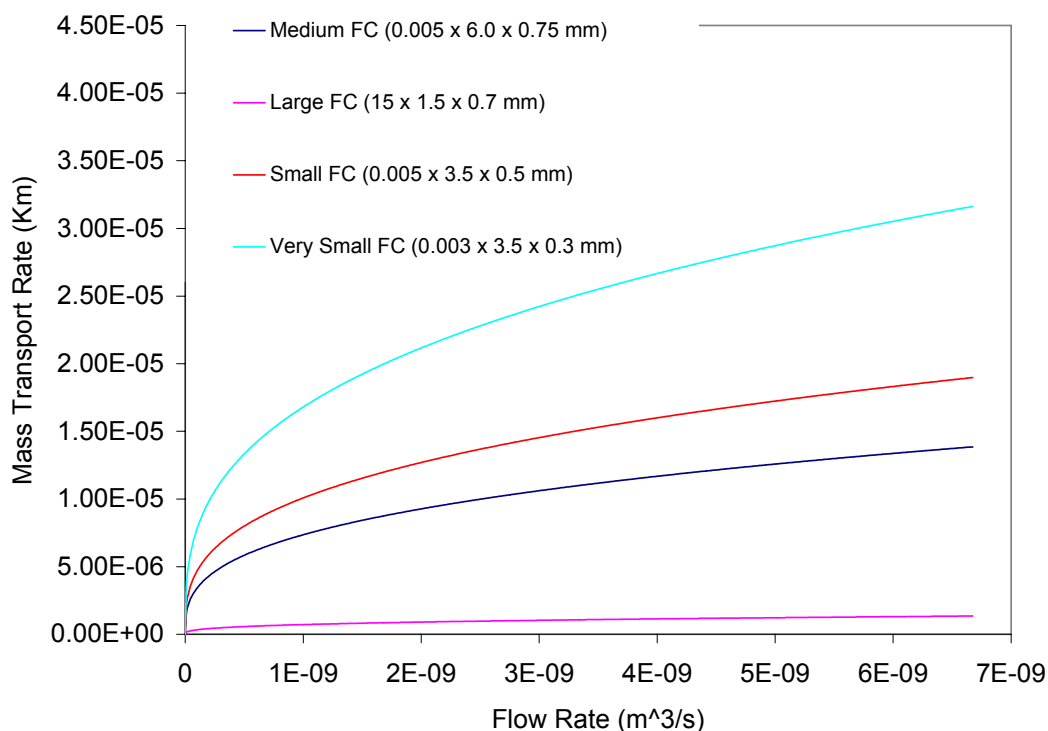
Figure 7. Relationship between flow rate and mass transport coefficient. It is obvious that small flow cells yield high mass transport rates and that reducing the height of the flow cell is very effective in increasing the mass transport rate. Obviously, concerns regarding clogging and backpressure place a limit on the height but a height of 50 μm is reliable.

In figure 7 it is obvious that mass transport rates are greatly influenced by the size of the flow cell and this has a large effect on the magnitude of binding signals that may be detected. In fact the situation is worse than figure 7 predicts as it is assumed that laminar flow is fully developed in all flow cells. In reality laminar flow will never be established in a flow cell with the dimensions of the large flow cell. Hence mass transport will be even lower than Figure 7 estimates. Figure 8 shows simulated interaction curves for each flow cell dimension. The large flow cell gives poor results while to other smaller flow cells perform well.
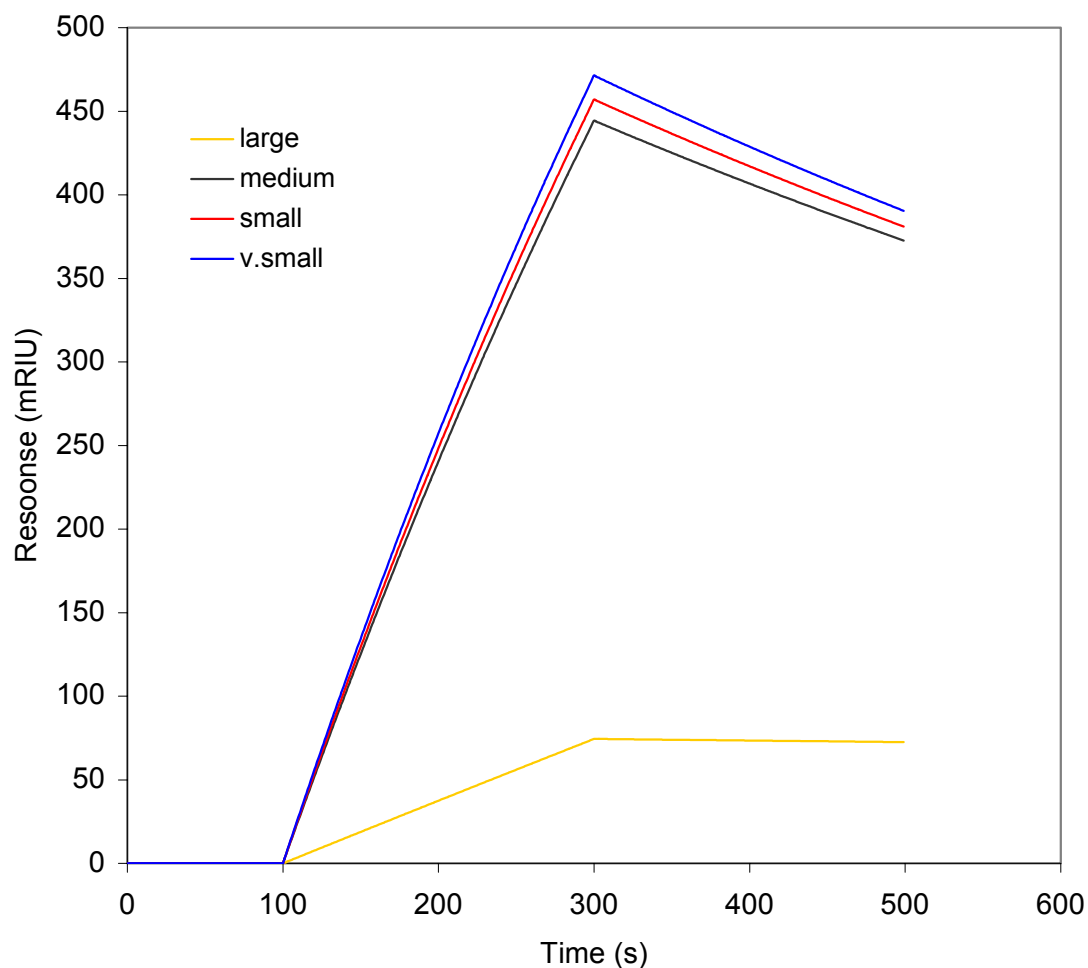
Figure 8. Simulated interaction curves for an analyte concentration of 1 nM. All parameters are kept constant with the exception of the flow cell geometry. Other constants used in this simulation are listed below. The very small, small and medium flow cells are comparable but the response for the large flow cell is several fold lower.

> *Flow rate = 30 μl/min.*
> *Contact Time = 200s.*
> *Analyte Molecular Weight = 150,000.*
> *Ligand Molecular Weight= 50,000.*
> *Immobilization Response = 1000 μRIU.*
> *Response Factor = 3.*
> *Temperature = 25 ℃.*
> *Data Collection Rate = 1 Hz.*
> *k_a = 1 x 10^6 M^{-1} s^{-1}.*
> *k_d = 0.001s*

In addition the smaller the flow cell area in contact with the sensing surface the greater the binding response observed. For example, under ideal conditions 100 pg of analyte binding to a 1 mm^2 surface will give a binding response of 100 μRIU. If the surface area was increased to 10 mm^2 then the binding response will fall to 10 μRIU due to distributing the sample over the greater surface. Therefore reducing this area to a minimal area encompassing the active SPR region is important.

Surface Chemistry

The surface chemistry employed will directly influence the binding capacity and non specific binding of the surface. There are many high tech and low tech approaches to surface chemisty but the best transducer available is worthless without an effective biointerfacial chemistry. If one is interested in improving the analyte limit of detection then it is necessary to maximize the concentration of ligand attached at the surface. When using an extended (e.g. 100 nm) hydrogel it is possible to load 10 monolayer equivalents of protein thus increasing the response 10 fold with respect to monolayer coatings. In addition proteins attached in this manner retain > 80% of their binding activity. In contrast it has been reported that antibodies absorbed as a monolayer onto plastic or metal surfaces lose 90% of their binding activity. Hence passive adsorption results in almost 100 fold less active binding sites as compared to the hydrogel method thus increasing the analyte limit of detection accordingly. Hence, the use of an anchor film to link ligands to the gold surface is essential in order to maintain high binding capacity. The commonly employed avidin-biotin system is simple and yet effective. In summery it is important to use log chain hydrophilic polymers when attaching molecules to the anchor film. This will ensure three dimensional access to the attached ligand. In addition, surfaces coated with hydrophilic polymers such as polyethyleneglycols (Shearwater Inc), or dextrans (Sigma-aldrich Inc), are excellent at resisting non specific adsorption of molecules.

See other application notes for details.

SAMPLING METHOD

The flow rate, contact time, non-specific binding, dispersion, and other system specific parameters will influence the limit of detection. Optimization of sampling methodology is essential. Dispersion occurs due to mixing of the running buffer with the sample before the sample reaches the flow cell. It is possible to limit dispersion by separating the sample from the running buffer within the flow channels by exploiting air bubbles. Minimizing the length of these channels is also effective.

Affinity

Assuming that all the above has been optimized it is only possible to obtain an analyte limit of detection that is within the range of the affinity of the analyte-ligand interaction. Hence care should be taken to ensure high affinity ligands are employed.

CONCLUSION

Quoting a molecular weight cut-off, or analyte limit of detection, is only applicable to the integrated SPR system that was used to record that data and is particular to that assay. When comparing Spreeta to other SPR devices it is the transducer performance that should be considered as the system it is integrated with is user dependent. However, the performance of Spreeta as a biosensor matches that of Biacore when equivalent peripheral technologies are employed. Therefore Spreeta users should familiarize themselves with these principles before conducting experiments under sub-optimal conditions. The crude flow cell available to customers is intended to allow users to begin using SPR quickly and familiarize themselves with the software. It was not intended as a final system component. Attention to the simple concepts outline here will ensure maximum performance.