

Lead identification and optimization in crude samples using label free resonant acoustic profiling[†]

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Detecting the molecular basis of protein–protein recognition is an essential element in understanding protein function because their ability to form specific complexes with other proteins underlies most cellular processes. The use of labels has limitations, such as changes to the binding kinetics due to the alterations in structure and function that occur with label addition, difficulty in detecting biochemical activities and the need for additional steps in assay development. These issues have driven the development of label-free formats for identifying the full range of biochemical activities.

Although optical-based systems dominate the label-free biosensor market, electrochemical, piezoelectric and acoustic devices represent similar but significantly less expensive alternatives. Acoustic biosensors have been employed in the label-free detection of an incredibly broad range of analytes, from interfacial chemistries and lipid membranes, to small molecules and whole cells.

Resonant acoustic profiling (RAP) technology offers label-free, real-time analysis of biomolecular interactions and offers an efficient way to optimize the development and production process of recombinant proteins. RAP measures only the physical binding events and is insensitive to refractive index and colour changes. This enables direct measurement in undiluted crude and complex samples, such as cell culture media or periplasmic extracts, without intensive assay calibration. This advantage simplifies experimental design and eliminates expensive time-consuming purification of often limited material, while delivering high content information. In this respect RAP technology reduces costs and increases the throughput and the density of information to optimize and control the processes more effectively. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords: BIA; biosensor; crude samples; label-free; real-time

INTRODUCTION

The widespread adoption of biosensors started in the early 1990s when the first optical platforms, based on surface plasmon resonance (SPR), entered the market. While advanced optical systems are the most widely used biosensors, there are also electrochemical-based biosensing devices, piezoelectric devices and acoustic devices, which represent similar but significantly less expensive alternatives. One type of biosensor uses Quartz Crystal Microbalance (QCM) technology. The popularity of devices using this technology is reflected in the number of publications that involve QCM, which have increased rapidly since the mid 1980s and in particular since the 1990s. Acoustic biosensors have been employed in the label-free detection of an incredibly broad range of analytes, from interfacial chemistries and lipid membranes to small molecules and whole cells (Marx, 2003; Skládal, 2003; Cooper and Singleton, 2007). A detailed overview of electrochemical biosensing devices and various combinations of electrochemical sensing with optical and acoustic-based sensing techniques and biosensor devices is given in a review by Grieshaber (Grieshaber *et al.*, 2008).

Resonant acoustic profiling (RAP) is an advanced label-free sensor technology that detects biomolecular interactions in real time (Godber *et al.*, 2005). RAP exploits the quartz crystal microbalance technique to analyse molecular binding and implements a number of technical advances. These include: the use of high fundamental frequency (16.5 MHz) resonators; submicroliter dead volume microfluidics; internal (on sensor) reference controls and a stress-free crystal mount that replaces the conventional O-ring

design used in other QCM devices. The new design markedly improves baseline stability enabling the study of slower off-rates and also creates a smaller flow cell for improved kinetics with higher sensitivity (Sota *et al.*, 2002). Furthermore, novel algorithms have been implemented for more accurate determination of the resonant frequency change to observe the molecular interactions. One such instrument that exploits RAP technology is RAPid 4, which has been designed to analyse up to 4 samples or combinations of samples and control materials in parallel, typically processing an average of 350 samples per day. The parallel inline reference controls in this instrument facilitate subtraction of background or

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[†] This article is published in *Journal of Molecular Recognition* as a special issue on Affinity 2009, edited by Gideon Fleming, Tel-Aviv University, Tel-Aviv, Israel and George Ehrlich, Hoffmann-La Roche, Nutley, NJ, USA.

Abbreviations: BSA, bovine serum albumin; DMEM, Dulbecco/Vogt modified Eagle's minimal essential medium; EDC, 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride; Fab, fragment antigen binding; FCS, fetal calf serum; HCl, hydrochloric acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; IgG, immunoglobulin; LB, lysogeny broth; MslgG, mouse immunoglobulin; NHS, N-hydroxysuccinimide; QCM, Quartz Crystal Microbalance; RaM-Fc, rabbit anti-mouse Fc-specific immunoglobulin; RAP, resonant acoustic profiling; RSD, relative standard deviation; SPR, surface plasmon resonance.

bulk (non-binding) signals that can often mask specific binding signals and as a fully automated platform, it requires minimal user intervention and runs unattended for days.

Acoustic biosensors offer substantial advantages over optical biosensors. One benefit is the reduction of the need for time-consuming and expensive sample purification. The direct detection of the association and dissociation of molecules on the surface of the quartz crystal enables the determination and quantification of molecular interactions in buffered solutions or in complex biological matrices that may contain organic solvents, serum, growth media or other impurities. Accurate kinetic, affinity and concentration measurements can be obtained and the collected data can be used to precisely determine the concentration of target molecules across a 3-log dynamic range thereby reducing re-testing. In a global benchmark study that explored the reproducibility of biosensor analysis participants from around the world, RAP technology was found to produce comparable data and rank highly among other biosensor instruments in determining the kinetic rate constants of a GST-fusion protein binding a fragment antigen binding (Fab) fragment (Rich *et al.*, 2009).

In addition to protein–protein interaction analysis, RAP technology can be used to characterize the binding of compounds as small as a few hundred Daltons and as large as whole cells (Huang and Cooper, 2006; Li *et al.*, 2006; Uludağ *et al.*, 2008; Natesan *et al.*, 2009). Currently, few technologies offer life scientists the ability to detect real-time kinetic data of this quality across such a broad range of samples, sample purities and concentrations.

MATERIALS AND METHODS

Instrumentation and sensors

RAP experiments were conducted using an automated four-channel RAPid 4 instrument in combination with covalent

sensors (TTP LabTech Ltd., Melbourn, UK). Sensors comprised standard gold-coated quartz wafers that are proprietary stress-free mounted in an acrylic cassette. The sensor surfaces used were coated with a proprietary planar, compact alkane chain linker layer terminated in carboxylic acid groups to provide a surface for protein immobilization.

Materials

The covalent sensor surfaces were activated using a 1:1 mixture of 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) (both from Thermo Fisher Scientific, Cramlington, UK). At the end of the immobilization cycle the remaining carboxy groups were capped using ethanolamine (Sigma–Aldrich, Poole, UK). The antibodies and antibody fragments used (rabbit anti-mouse Fc-specific immunoglobulin (RaM-Fc), mouse immunoglobulin (MslgG) or mouse immunoglobuline (IgG) Fab-fragment) were from Jackson ImmunoResearch, Soham, UK. The capture surfaces were prepared using protein A and protein L (TTP LabTech, Melbourn, UK). All other reagents were supplied by Sigma–Aldrich, Poole, UK.

Sensor surface preparation

Sensor surfaces were activated with a 1:1 mixture of 400 mmol/l EDC and 100 mmol/l NHS, prepared in 0.22 μm filtered deionized water and mixed immediately prior to use (final concentrations: 200 mmol/l EDC and 50 mmol/l NHS). EDC-NHS was injected simultaneously across both sensor surfaces for 3 min. Protein A, protein L or specific antibodies were prepared for immobilization at 50 $\mu\text{g}/\text{ml}$ in immobilization buffer comprising 10 mmol/l sodium acetate, pH 4.5 or 5.5, and were injected simultaneously across separate sensor surfaces for 3 min. Control surfaces were prepared in parallel using a non-specific MslgG or bovine serum albumin (BSA). Non-reacted NHS esters were then capped with 1 mol/l

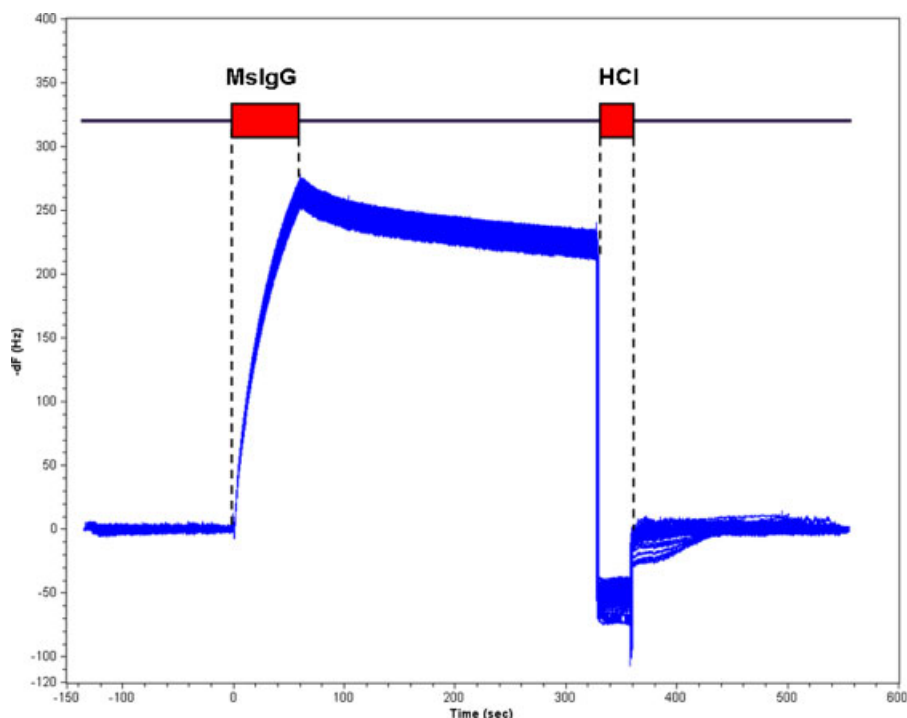


Figure 1. Protein A surface stability test using immunoglobulin injections. The plot shows the overlay plot of 60 s injections ($n = 240$) of a mouse IgG at a fixed concentration of 25 $\mu\text{g}/\text{ml}$ to test the stability of the surface. The surface was regenerated in between using a 30 s injection of 5 mM HCl.

ethanolamine prepared in 0.22 μm filtered deionized water, pH 8.5. The running buffer used between sample injections was Dulbecco's modified phosphate buffered saline, at a flow rate of 25 $\mu\text{l}/\text{min}$.

Data analysis

The data generated were used for sensor surface characterization. The data were plotted and analysed using the RAP

Workbench data analysis software (TTP LabTech, Melbourn, UK). To obtain sensorgrams that represent the specific binding of analyte to immobilized ligand, the response from a reference channel (containing non-specific immunoglobulin or BSA) and the response from a blank buffer injection were subtracted from the entire data set (termed 'double referencing') (Myszka, 1999). The double reference control subtracted data were used for

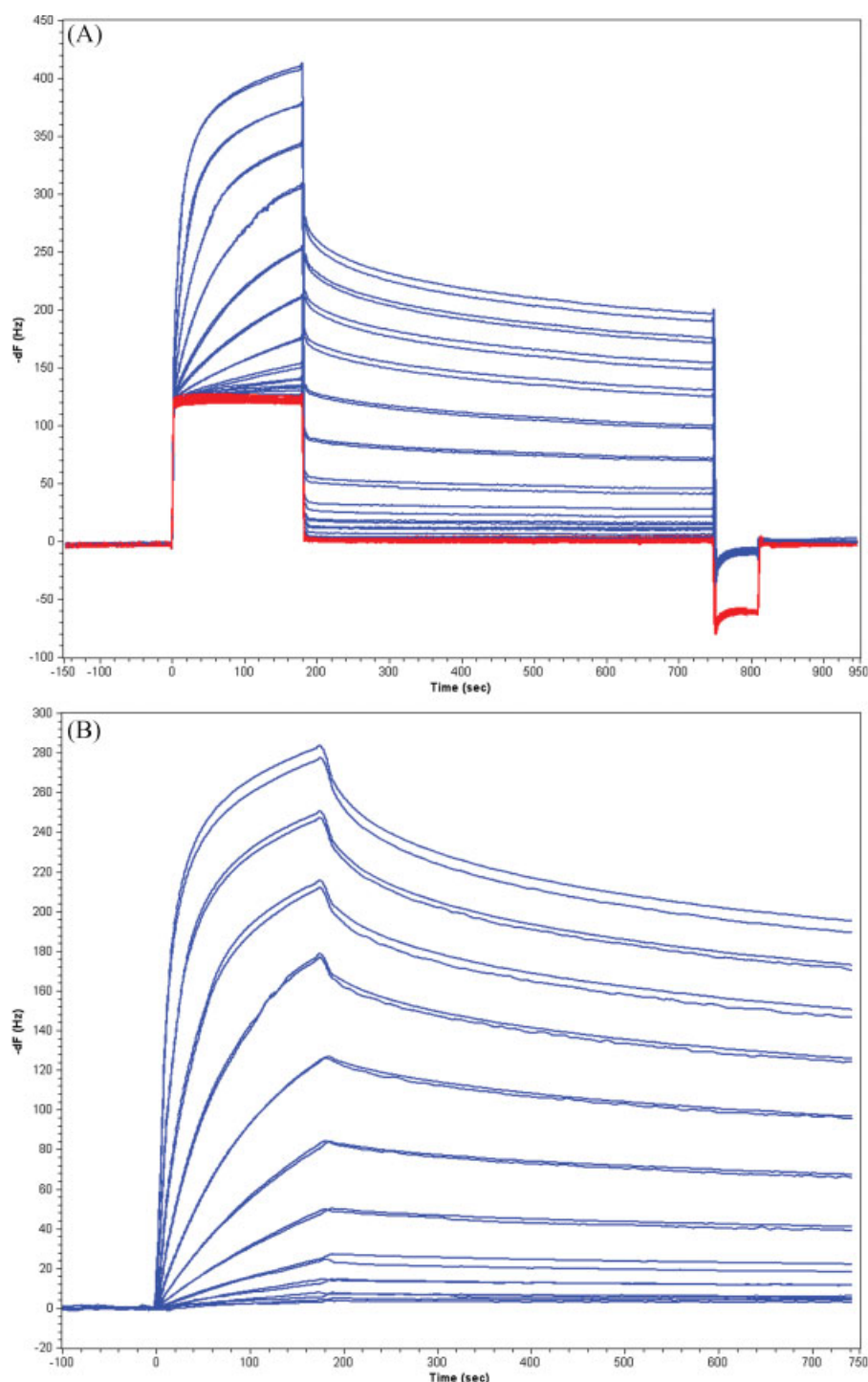


Figure 2. Antibody determination in undiluted culture media. (A) The raw data of a dilution series of human IgG in 100% LB media were collected in an active (blue) and a control channel (red) in parallel. A bulk shift was observed due to a change in viscosity. (B) The raw data were double reference subtracted, before further data processing. (C) The initial binding rate was calculated and plotted vs. concentration to generate a calibration curve to determine the concentration of unknown samples.

analysis. Initial binding rates and the binding levels were determined by calculating the Hz/s during the first 30 s of the injection or the binding response during the first 20 s of the dissociation, respectively.

RESULTS AND DISCUSSION

Protein A

Protein A was tested as an inexpensive alternative to an antibody capture assay. The Protein A assay for determining protein concentration is based on the rate of binding of a protein of interest to the biosensor surface. The initial binding rate,

proportional to concentration, is determined using the first 30 s of the binding event. A fixed concentration of a mouse IgG (10 μ g/ml) was used to test the stability of the surface. Figure 1 shows the plot of consecutive 60 s injections ($n = 240$). The surface was regenerated in between using a 30 s injection of 5 mM hydrochloric acid (HCl). Initial binding rate and binding response level (first 20 s of the dissociation) were determined as quality criteria of the reproducibility of the assay. The average of the initial binding rate was determined to 10.3 ± 0.6 Hz/s (% RSD 5.8). The average binding level was 249.7 ± 7.3 Hz (%RSD 2.9).

A human IgG was diluted in 100% lysogeny broth (LB) media and directly injected. A bulk shift was observed due to a change in viscosity. However, the RAP biosensor is not sensitive to refractive index changes. This allows the direct detection of

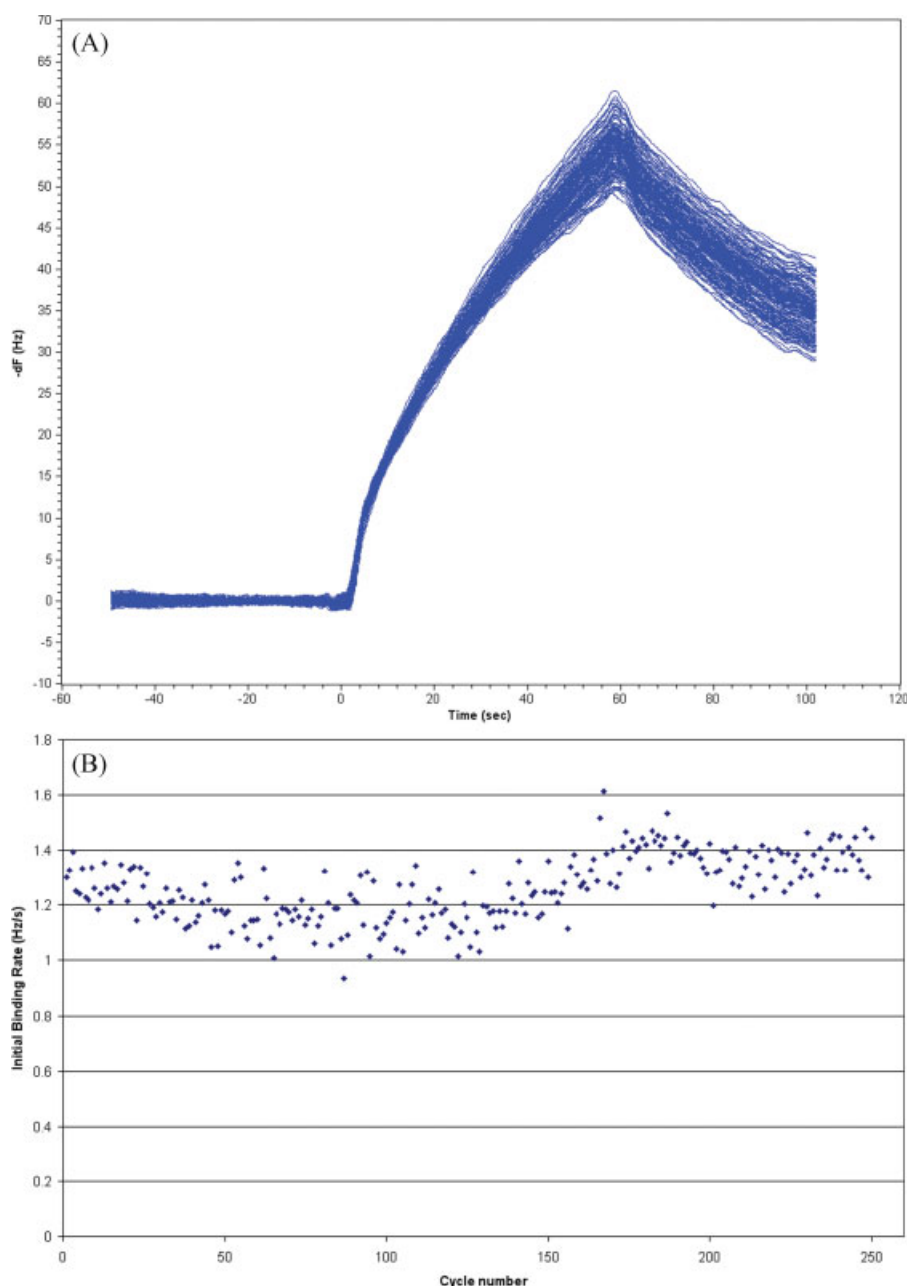


Figure 3. Protein L surface stability test using a Fab fragment. (A) The plot shows 60 s injections ($n = 252$) of a mouse Fab fragment at a concentration of 10 μ g/ml to test the stability of a protein L surface. The surface was regenerated in between using a 15 s injection of 1 mM HCl. (B) The initial binding rate was determined as quality criterion of the reproducibility of the assay. The initial binding rate was plotted versus cycle number.

binding in crude or non-purified samples without further sensor calibration and eliminates expensive time-consuming purification of often limited material while delivering high content information. Figure 2 shows the 3-step data evaluation process of collecting the raw data, reference subtraction and generating the standard curve to determine unknown samples.

A protein A biosensor was successfully validated for use with the RAP platform. The biosensor surface can routinely be

used to monitor antibody concentrations or as a capture surface for kinetic characterizations. Different protein concentrations result in different initial binding rates and the binding rates from standards with known concentrations can be used to generate a standard curve. Concentrations of experimental samples can be calculated based on their binding rate compared with that of the standard curve. The surface stability was seen to be excellent and lasted for at least 1000 sample injections

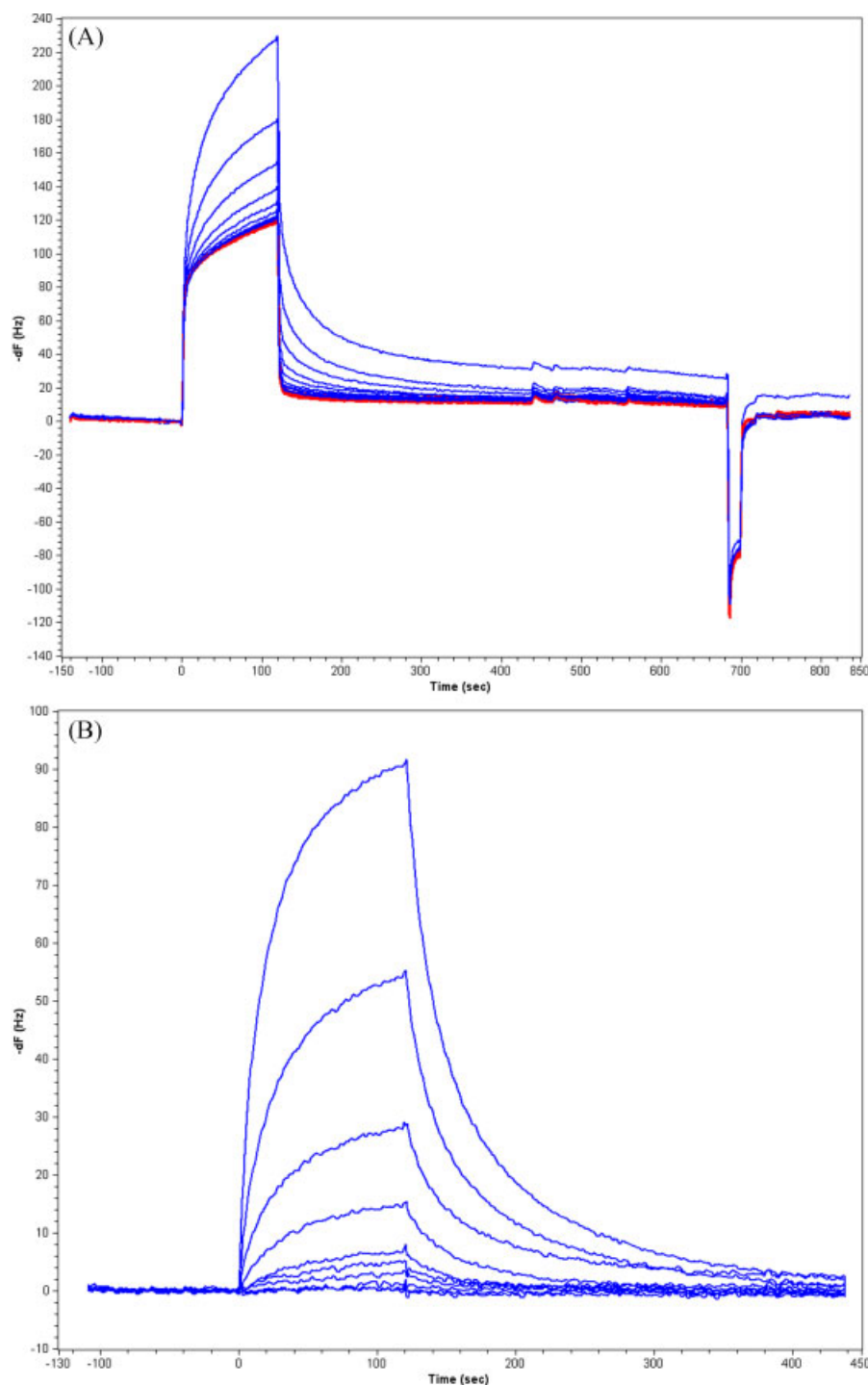


Figure 4. Determination of a Fab fragment in undiluted DMEM media containing 10% FCS. (A) Raw data of Fab fragment diluted in culture supernatant samples (DMEM containing 10% FCS) binding to a protein L surface (blue). The concentration range measured was between 100 and 0.2 $\mu\text{g/ml}$. A bulk shift was observed due to a change in viscosity (red). (B) The data shown are double reference subtracted for further data processing.

(four channels in parallel) before the sensor surface needed to be recalibrated.

As the RAP biosensor is not sensitive to refractive index changes it enables the direct detection of binding in crude or non-purified samples without further sensor calibration. This eliminates expensive time-consuming purification of often limited material while delivering high content information. In addition, antibodies can be arrayed by using protein A covalently attached to the sensor as capture surface. A ligand antibody is bound to the protein A surface in a preliminary step followed by the analyte injection. An advantage of the protein A surface is that complete surface regeneration can be achieved by using very mild regeneration conditions even for high affinity binding interactions.

Protein L

Protein L was tested as an inexpensive alternative to an antibody capture assay. Figure 3 shows consecutive injections of one concentration of a mouse Fab fragment (10 µg/ml) to test the

stability of the surface ($n = 252$). The surface was regenerated inbetween using a 15 s injection of 1 mM HCl. The initial binding rate was determined as quality criterion of the reproducibility of the assay. The average was 1.3 ± 0.1 Hz/s (%RSD 9.4).

A mouse Fab fragment was diluted in Dulbecco/Vogt modified Eagle's minimal essential medium (DMEM) containing 10% fetal calf serum (FCS) as a model for a hybridoma supernatant. The samples were injected undiluted over an active and a control channel in parallel (Figure 4).

A protein L biosensor was successfully validated. Protein L can bind a wider range of Ig classes through interactions with the kappa light chain and can bind single chain variable fragments and Fab fragments. Protein L was not seen to bind bovine immunoglobulin, present in the media serum supplement, and does not interfere with the antigen-binding site of the antibody (capture assay ability).

The biosensor surface can routinely be used to monitor antibody or antibody fragment concentrations from undiluted crude samples or as a capture surface for kinetic characterizations. The surface stability was shown to be excellent and lasted

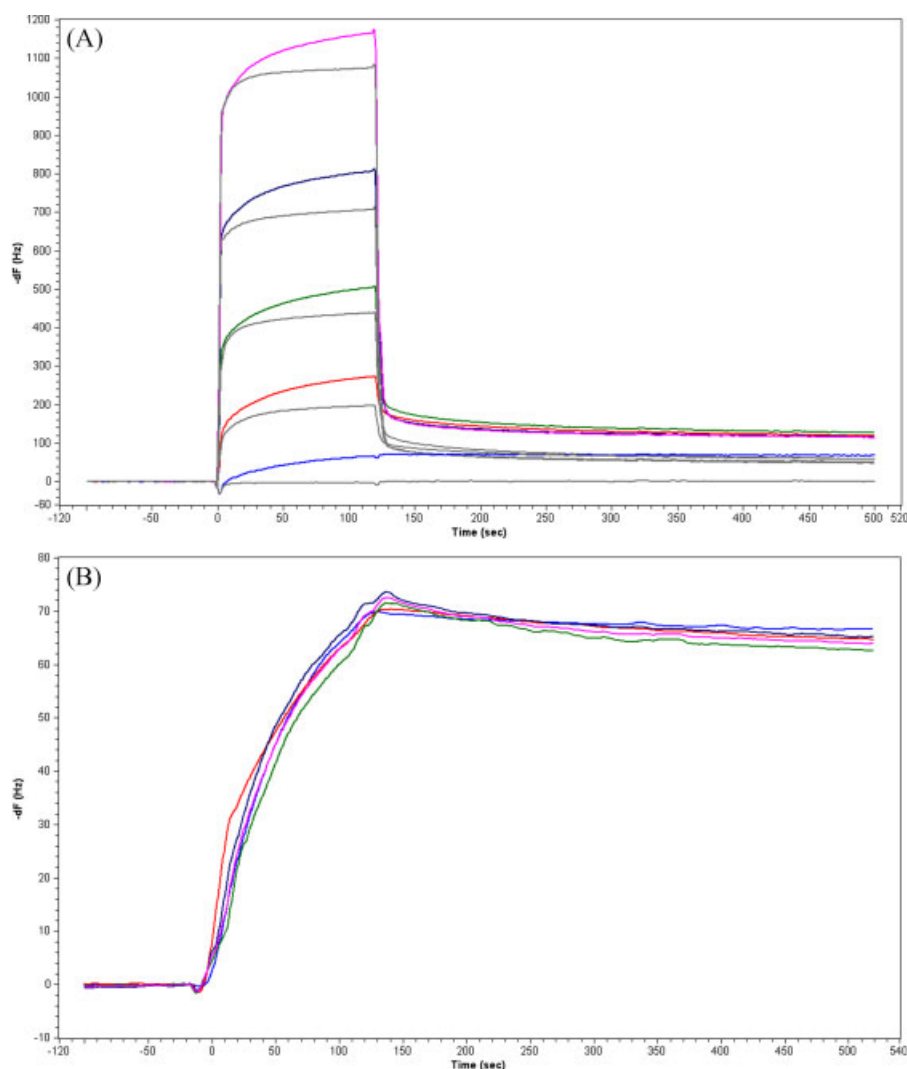


Figure 5. Determination of immunoglobulin in the presence of high concentrations of human serum. (A) Raw data of human IgG binding to a specific anti-human antibody in HEPES buffer, in 10, 25, 50 and 75% human serum. Increasing the serum content of the sample increased the observed bulk shift. (B) Control subtracted data of the binding interactions. Virtually no difference was observed between the binding signals in buffer or in high levels of human serum (up to 75%).

for at least 1000 sample injections (four channels in parallel) before the RSD exceeded 10%.

Serum samples

The following preliminary work was performed to demonstrate the capability of the RAP biosensor for real serum sample examination. RAP technology was successfully used to detect protein-protein interactions in samples containing high levels of human serum. Figure 5 shows preliminary data of human IgG binding to a specific anti-human antibody in up to 75% human serum. A constant concentration of human IgG (10 µg/ml) was spiked in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (control), 10, 25, 50 and 75% human serum and injected over the active and a control channel (MslgG) in parallel.

Biological and biotechnology-derived proteins are increasingly used as therapeutic agents. These products may induce an unwanted immune response in treated patients, which can be influenced by various factors, including patient- or disease-related factors and product-related factors. The consequences of such immune reactions to a biotherapeutic, range from transient appearance of antibodies without any clinical significance to severe life threatening conditions. Therefore, it is essential to adopt an appropriate strategy for the development of adequate screening and confirmatory assays to measure an immune response against a therapeutic protein. Protein-protein interactions are affected by the environments that they are studied in, thus the ability to study these effects at physiological temperature and in a physiological milieu, such as serum, enables the generation of more biologically relevant data than has previously been permitted using biosensors.

CONCLUSIONS

RAP provides high sensitivity and the ability to detect analytes in complex matrices as a result of the improvements that have been made in surface chemistry, fluidics, higher frequency crystal and flow cell design.

With its flexible assay design due to a variation of sensor surface attachments and binding chemistries, RAP biosensors can be used to analyse a wide range of molecular interactions. Its use can extend from research, through biotherapeutic development, protein expression analysis into preclinical studies and includes the following key applications:

- Optimization of recombinant protein and antibody affinities as biopharmaceuticals
- Assessment of the concentration and integrity of proteins
- Immunogenicity studies
- Improvement of protein expression, extraction and purification procedures
- Quality control of production processes in manufacturing

RAP biosensors can be used but are not limited to the screening and kinetic analysis of monoclonal antibodies straight from cell culture supernatants, screening for recombinant proteins or biomarkers directly in expression media such as *Escherichia coli* or Baculovirus media, and can be used for concentration determination of recombinant proteins in periplasmic extracts. Furthermore, with RAP biosensors it is possible to detect immune response to a protein therapeutic in patient serum samples and determine both the isotype and concentration of the anti-protein therapeutic immunoglobins.

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