Chapter 6

Label-Free Detection with the Resonant Mirror Biosensor

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Summary

The resonant mirror (RM) biosensor is a leaky waveguide-based instrument that uses the evanescent field to probe changes in the refractive index at the sensing surface.The RM can therefore be used to monitor in real-time and label-free the interaction between an analyte in solution and its biospecific partner immobilized on the waveguide surface.The RM has been used in studying the interaction of a variety of moieties including proteins, carbohydrates, cells, nucleic acids and receptors, leading to applications in areas such as clinical diagnostics, homeland security, and pharmaceutical and biomolecular interactions. This chapter will review the principle of this biosensor, and the recent advances in instrumentation, different immobilization chemistries, and kinetic studies, as well as some applications.

Key words: Resonant mirror biosensor, Leaky waveguide biosensor, Label-free. Biomolecules immobilization, Refractive index, Immunosensors, Cell detection, Nucleic acid hybridization, Kinetics, Biomedical application, Industrial applications.

1. Introduction

Optical evanescent biosensors greatly facilitate the study of the interaction between a range of interactants. The resonant mirror was developed in 1993 *(1, 2)* and demonstrated in a number of applications, including concentration determination *(2)*, molecular recognition *(3, 4)*, kinetics association and dissociation *(5, 6)*, epitope mapping for different analytes including proteins *(7–9)*, nucleic acids *(10–12)*, carbohydrates *(13–16)*, cells *(17–19)*, and receptors *(20, 21)*, in addition to industrial applications *(22–25)*. In principle, the user links one of the interactants to the sensor

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surface, adds the other reactants and follows the binding response. The optical signal is generated by the change in the refractive index as the binding species displace the water molecules from the interrogated surface. It will provide information about the strength and rate of binary complex formation and dissociation. This chapter will cover some background about the resonant mirror, recent developments, different protocols used for immobilizations, and some applications and kinetics measurements.

1.1. The Resonant Mirror Instrument

1.1.1. The RM Structure

The resonant mirror (RM) was first commercialized by Affinity Sensors (IAsys, Cambridge, UK), then Thermo Electron acquired the technology, becoming Thermo Labsystems, and now it has been transferred to NeoSensors (Durham, UK). In the RM sensor, FTIR is used to couple the light in and out of a high-index waveguiding layer. The RM is effectively a prism coupler where the air gap has been replaced by a low-index dielectric layer. **Figure 1** shows the RM device integrated with a microfluidic and stirrer. The RM chip structure, consists of a high-index substrate prism (n = 1.72), a thin low-index spacer (about 550 nm of silica, n = 1.45) and a very thin monomode waveguiding layer (about 80 nm of Si_3N_4, n = 2.0) *(1, 2)*. The high-index resonant layer acts as both a waveguide and the sensing layer. The light incident above the critical angle on the substrate–spacer interface is coupled with the waveguiding layer via the evanescent field in the spacer, when the propagation constants in the substrate prism and the waveguide match. For monochromatic light (angular scan

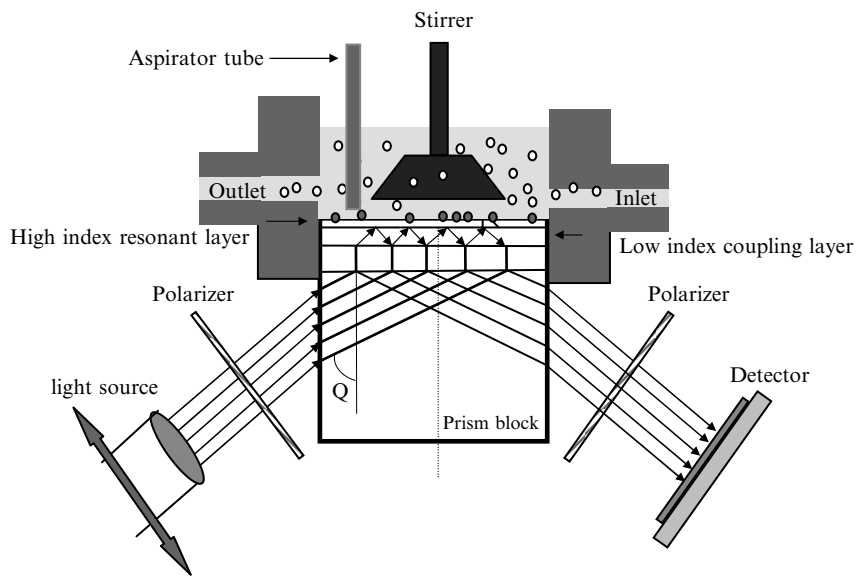


Fig. 1. Schematic showing the structure of the resonant mirror integrated in a microfluidic and stirrer.

using constant wavelength), this occurs over a very narrow range of incidence angles, typically spanning considerably less than 1°. The light decoupled out via the coupling layer emerges to strike the detector. The resonance angle is very sensitive to the change in the refractive index within the evanescent field at the surface and will not detect changes in the bulk outside the evanescent field. Thus by immobilizing recognition receptors (ligand), it is possible to measure only those molecules (ligates) that bind to or dissociate from the ligand. To resolve the resonance angle, the optical components are arranged so that a 90° rotation of polarization occurs for the light to travel along the waveguide. Another polarizer is placed in front of the detector to enable only the light that has traveled along the waveguide to reach the detector. A sharp peak of intensity is seen at the discrete angle of interest and this is extremely sensitive to the surface binding/dissociation.

In the IAsys the laser light is scanned over an angle of 14°, the middle 10° of which is used for detection purposes. The other 4° is used to ensure the laser is moving at a constant speed over the measurement region. An encoder grating located on the end of the laser arm enables the position of the arm to be known at any time. The time at which the light falls on the detector is monitored and converted into a resonance angle, the resulting sensorgram displaying the resonance angle vs. time in the IAsys software *(25)*.

The resonant mirror can be operated using two instrumental configurations, the angular scan at constant wavelength **(Fig. 2a)** and wavelength scan at constant angle **(Fig. 2b)** *(26)*. It was shown that the two configurations of the RM (the angular scan at constant wavelength and wavelength scan at constant angle) have equal sensitivity in biosensing *(26)*. The choice of the operating mode is governed more by the availability of suitable light sources and detectors than by any fundamental difference between the two modes of operation. Goddard et al. demonstrated a grating modified resonant mirror for chemical/biosensing application *(27)*. This was achieved by fabricating a grating (closely-spaced strips) parallel to the direction of the incoming light, which allows miniaturization of the RM optical setup and eliminates the need for polarized light, as on resonance there is a π phase difference between the light reflected from the stripes and the area between the stripes *(27)*. However, the IAsys instrument commercially available uses the prism coupling and angle scanning mode (monochromatic light).

The resonant mirror modes are dispersive as well as leaky, causing the coupling angle for a particular mode to shift as the illumination wavelength changes. This is a particular problem when using coherent illumination light sources e.g., laser diodes, as these are prone to mode hopping as the diode temperature changes. This leads to sudden changes in the wavelength of the

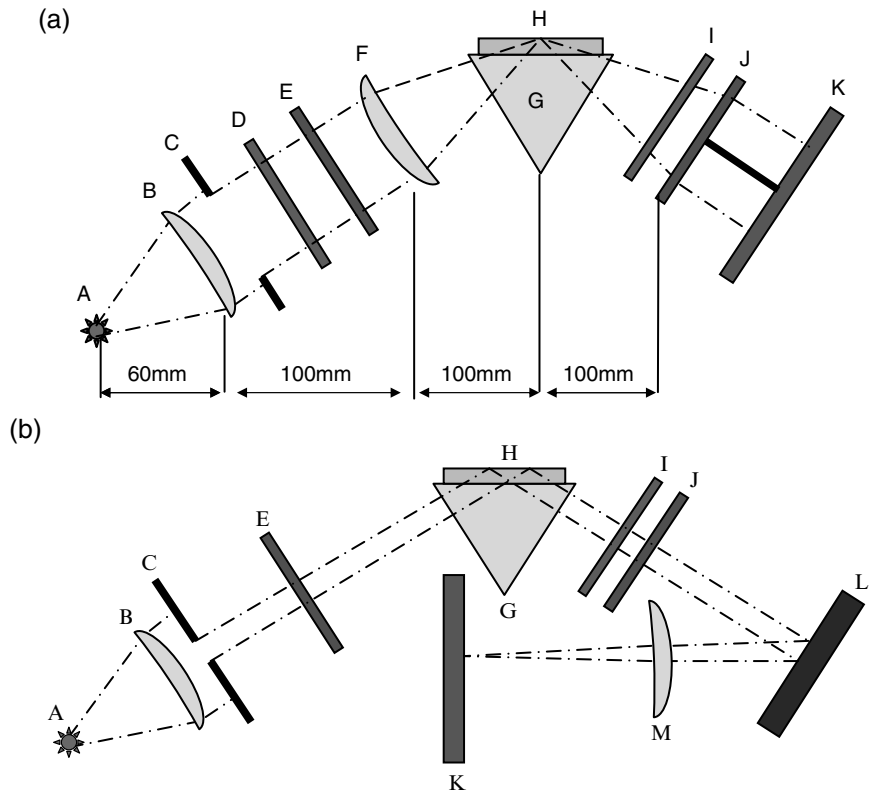


Fig. 2. Schematic diagram of the resonant mirror configurations for (**a**) angular scan (**b**) wavelength scan. A light source (LED or tungsten lamp); B collimating lens; C aperture stop; D filter; E input polarizer; F plano-convex cylindrical lens; G equilateral SF 10 prism; H RM sensor chip; I quarter wavelength plate; J output polarizer; K detector; L grating; and M collimating lens.

laser output, causing a similar abrupt change in the measured resonance angle. The internally-referenced resonant mirror has been developed to overcome problems in changing illumination wavelength *(28, 29)*. In this approach, an additional buried RM waveguide layer was incorporated into the sensor structure. The buried waveguiding layer provides a second resonance that has the same sensitivity to wavelength changes as the conventional surface RM resonance, but is considerably less sensitive to surface refractive index changes *(28, 29)*.

1.1.2. The Sample Cuvette

The IAsys resonant mirror chip is mounted onto a cuvette structure such that the sensing surface is exposed to the sample contained within the cuvette lumen (**Fig. 1**). The sample volume within the cuvette chamber for single channel is approximately 200 µL and 80 µL for the dual channel. The cuvette body is coated with a biocompatible coating to prevent the non-specific binding. A vibro-stirring mechanism and a vacuum aspiration system are

inserted into the cuvette after placement in the instrument. The vibro-stirrer is used to ensure efficient mass transport of materials to the sensor surface and rapid equilibration of the system. As the stirrer vibrates, liquid is forced towards the surface of the sample chamber and back up through the centre of the stirrer cone. The stirrer frequency is fixed at 126 Hz but it can be adjusted via the instrument operating software. Reagents and samples can be added either by a pipette or by a syringe using the auto-sampler. The samples can also be removed either by a pipette or by the vacuum-driven aspiration systemto waste bottles.

As the refractive index measurements are temperature sensitive, the cuvette is clamped tightly against peltier-driven thermal pads when inserted in the instrument. Good thermal conductivity is ensured between the peltier pads and the sample by the configuration of the cuvette body. Thermal equilibration time is typically 5–10 min for a temperature change of + or –10 °C. The rapid temperature equilibration allows the real-time study of the effect of temperature in binding studies. The use of real-time reference signal subtraction in the RM experiments allows the elimination of baseline shifts due to operating temperature changes. Furthermore, temperature changes can be easily incorporated into IAsys auto + scripts. The use of the unique real-time decision making function (ASK) allows the IAsys software to wait for complete baseline re-equilibration to be established before continuing an experiment.

1.1.3. Data Acquisition

IAsys machines have two data acquisition software, FASTplot and GraFit, which enable rapid and comprehensive analysis of data. It has a number of templates that can be used for kinetic and affinity analysis of data obtained from the IAsys. The supplied templates enable users to go from binding curves to values for affinity and kinetic constants easily. In-built flexibility enables users to modify existing templates, for example, constraining a fit to a value of k_{diss}, to creating their own templates. The templates make use of hard coded equations, which are also supplied as separate items, to simplify the fitting. Using 'hard-coding' removes the requirement on users to supply initial estimates to the non-linear fitting engine. FASTplot enables the relevant data from one or more experiments to be accumulated. Coupled with the latest versions of the operating software, automatic region selection can be obtained from the Event log. Concentrations entered during the experiment are carried through, speeding up the processing. Linear correction to baselines can be applied to the data during the accumulation, and subtraction from control experiments can also be performed in FASTplot, prior to the data analysis. Once the data have been accumulated, the data to be fitted can be rapidly exported directly into a ready-made fitting template in GraFit, and results generated without further

user input. GraFit also enables users to generate their own templates and analysis methods. GraFit (Erithacus software) is a fully-functioning, spreadsheet-based graphing and data analysis software.

1.1.4. Sensitivity

IAsys instruments measure the mass bound at the sensor surface; therefore the sensitivity can be considered in terms of the lowest amount of mass detectable per unit area. From the calibration factors (200 arcsec = 1 ng/mm^2 for matrix surfaces and 600 arcsec = 1 ng/mm^{2}for planar surfaces) and the signal to noise, it is possible to calculate the sensitivity to mass of the IAsys range of instruments. As mentioned above, the minimum sensitivity of the system can be considered in terms of the minimum surface concentration that can be resolved. This will depend upon the smallest response that can be detected over and above that of the baseline. The latest range of IAsys instruments can be divided into two types, standard and enhanced. One source of noise is caused by disturbances to the laser light incident on the resonant mirror structure. The enhanced range of instruments have modifications that fully enclose the input light, shielding it from external influences such as convection currents, thereby reducing the background noise.

From the calibration factors and the arc second response corresponding to two times the standard deviation of the baseline noise, we can calculate the minimum detectable surface concentration of a protein. With the matrix cuvettes the detection limit of the standard instrument is 0.4 pg/mm^2 and that of the enhanced instrument is 0.1 pg/mm^2. With the planar surfaces the minimum detection level is 0.14 pg/mm^2 for the standard instrument and 0.04 pg/mm^2 for the enhanced instrument. It is however important to remember that the matrix surface is three-dimensional, and so in absolute terms will have a higher binding capacity per unit area of surface.

As the detection method used in IAsys is mass based, the larger the molecule, the larger the response. This means that in order to directly detect small molecules (100s of Daltons molecular weight range), a larger number of binding sites and higher solution concentrations are required. Competition studies can be performed in which a small molecule is allowed to compete against a conjugate of the small molecule coupled to a large molecule such as a protein. The binding of the protein conjugate is then detected, giving an enhanced response. It is possible to 'magnify' a response by building up a complex on the sensor surface, increasing the apparent bound mass and thereby increasing the sensitivity of the measurement. This is possible by using a sandwich molecule, for instance, an antibody to a second epitope on the target molecule. The signal can be further enhanced if the second antibody is labeled with colloidal gold. The potential gain in sensitivity, in this case approximately 1,000-fold,

in using colloidal gold-labeled secondary antibodies is shown in **Subheading 3.17**.

1.1.5. Resonant Mirror Surfaces

To ensure the specificity of the measurement, a recognition species (ligand) is immobilized at the sensor surface. To facilitate the immobilization, the IAsys RM chips have been derivatized with different matrices, e.g., 3D-carboxy methyl dextran hydrogel layer, and planar surfaces derivatized with amino, carboxylate, biotin, or hydrophobic groups. To ensure efficient mass transport of materials to the sensor surface and rapid equilibration of the system, a vibrating stirrer assembly is inserted into the IAsys cuvette after placement in the instrument.

2. Materials

2.1. Components for RM Setup

1. 670 nm laser diodes or high-intensity red light emitting diodes.
2. 20 W tungsten–halogen lamp.
3. Plano-convex with 25 mm diameter, EFL: 60 mm (45-509, Edmund Optics, York, UK).
4. 350 µm pinholes (53 mm outer diameter and 1.2 mm minimum aperture (32-618, Edmund Optics, York, UK).
5. Input-polarizer with 25 mm diameter (47-216, Edmund Optics, York, UK).
6. Plano-convex cylindrical lens with 25 mm diameter, EFL 100 mm (46-018, Edmund Optics, York, UK).
7. SF10 prism (n = 1.72, Comar instruments, Cambridge, UK).
8. Index-matching fluid (Cargille Labs Refractive Index Liquid Series M RI 1.730).
9. Output polarizer with 25 mm diameter (47-216).
10. Convex lens with 25 mm diameter, EFL: 60 mm (45-509, Edmund Optics, York, UK).
11. Grating (a 1,200 lines/mm replicated Ruled grating (36-4323) supplied by Ealing Electro-Optics (Watford, Hertfordshire, UK).

2.2. Materials for the Preparation of Dextran Coated Surfaces and Immobilization of Amine-Containing Ligand to CMD

1. Dextran MW 45,000 (Sigma)(*see* **Note 1**).
2. 0.1 M and 2 M sodium hydroxide.
3. 1 M bromoacetic acid.
4. 500 mL phosphate buffered saline pH 7.4, 0.05% Tween 20 (PBST).

5. EDC stock (1.15 g in 15 mL high-purity water), (*see* **Note 2**).
6. NHS stock (200 mg in 15 mL high-purity water, (*see* **Note 2**).
7. 5 mL 10 mM sodium acetate, pH 5.0.
8. 200 µL 0.05 mg/mL IgG in PBS. Storage buffers must be free of reducing agents, Tris and azide.
9. Blocking agent: 5 mL 1 M ethanolamine, pH 8.5; or 1 mM Tris–HCl, pH 8.0.
10. 25 mL 10 mM HCl.

2.3. Materials for Immobilization of Thiol-Containing Ligand to CMD

1. CMD chip (Neosensors, Durham, UK, CUV991100).
2. 500 mL 0.01 M phosphate buffered saline pH 7.4, 0.05% Tween 20 (PBST) (Sigma, P3563).
3. EDC stock (1.15 g in 15 mL high-purity water), (*see* **Note 2**).
4. NHS stock (200 mg in 15 mL high-purity water), (*see* **Note 2**).
5. 1 M ethylenediamine, pH 8.5 (Sigma, E1521).
6. 1 mg/mL Sulfo-SMCC in PBS (Pierce, 22322).
7. 1 mg/mL Cys-GM-CSF, thiol containing peptides in PBS.

2.4. Materials for Polymerizing the Glutaraldehyde and Immobilization on Amino-Silane Functionalized Surface

1. Amino-silane (AS) chip (Neosensors, Durham, UK, CUV991400).
2. 5 mL of 5% (v/v) glutaraldehyde (Sigma, G5882).
3. 500 µL 0.1 M NaOH.
4. 500 µL 0.1 M HCl.
5. 500 mL phosphate buffered saline pH 7.7 (PBS).
6. 200 µL 1 mg per/mL Rabbit anti-mouse Fc (RAMFc) (ICN Flow).
7. 25 mL 1 M formic acid.
8. 500 µL 1 mg/mL BSA in PB.

2.5. Materials for Immobilization of Amine-Containing Ligand to Carboxylate Surface

1. Carboxylate functionalized chip (Neosensors, Durham, UK, CUV991300).
2. 500 mL of phosphate buffered saline (PBS) pH 7.4,without Tween.
3. EDC stock (1.15 g in 15 mL high-purity water), (*see* **Note 2**).
4. NHS stock (200 mg in 15 mL high-purity water), (*see* **Note 2**).
5. 5 mL 10 mM sodium acetate, pH 5.5.
6. 200 µL of 1 mg/mL Rabbit anti-mouse Fc (RAMFc). Storage buffers must be free of reducing agents, Tris and azide.
7. 200 µL of 2 mg/mL β-casein (Sigma, C6905).
8. 25 mL of 1 M formic acid.

2.6. Materials for Immobilization of Thiol-Containing Ligand to Carboxylate Surface

1. Carboxylate functionalized chip (Neosensors, Durham, UK, CUV991300).
2. 500 mL phosphate buffered saline pH 7.4, (PBS).
3. 10 mL phosphate buffered saline pH 7.4, 0.05% Tween 20 (PBST).
4. EDC stock (1.15 g in 15 mL high-purity water), (*see* **Note 2**).
5. NHS stock (200 mg in 15 mL high-purity water), (*see* **Note 2**).
6. 5 mL of 1 M ethylenediamine, pH 8.5 (Sigma, E1521).
7. 5 mL of 1 M Tris pH 8.5.
8. 1 mg/mL Sulfo-SMCC in PBS (Pierce, 22322).
9. 1 mg/mL Cys-GM-CSF, thiol-containing peptides in PBS.

2.7. Materials for Immobilization of Avidin and Biotinylated Ligand Capture

1. Biotin functionalized chip (Neosensors, Durham, UK, CUV991200).
2. 500 mL phosphate buffered saline (PBS) pH 7.4, 0.05% Tween 20 (PBST).
3. 200 µL of 2 mg/mL avidin (Pierce, 21121) dissolved in high-purity water.
4. 200 µL of 0.2 mg/mL biotinylated protein G (Pierce, 2988) dissolved in high-purity water.
5. 200 µL of 0.1 mg/mL IgG dissolved in PBST, pH 7.4.

2.8. Materials for Immobilization of Thiol Containing Ligands to Avidin

1. Biotin functionalized chip (Neosensors, Durham, UK, CUV991200).
2. 500 mL of 20 mM HEPES-KOH, 138 mM NaCl, 2.7 mM KCl, 2 mM EDTA, pH 7.4 plus 0.05% Tween-20. (HSTE).
3. 100 µL of 5 mg/mL avidin (Pierce, 21121) dissolved in high-purity water. Streptavidin and NeutrAvidin from Pierce also can be used instead of avidin.
4. 500 µL of 50 mM *N*-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) (Sigma, P-3415 or Pierce, 21857) dissolved in DMSO.
5. 5 mL of 10 mM sodium acetate pH 5.5.
6. 25 mL of 100 mM HCl.
7. Reduced and thoroughly desalted IgG at 0.1 mg/mL was used for this example. Storage buffer must be free of reducing agents, Tris and azide.
8. 2 mg/mL IgG dissolved in HBSTE.
9. D-salt Dextran plastic desalting column (Pierce, 43230).

2.9. Materials for Immobilization of Amine Containing Ligands to Avidin

1. Biotin functionalized chip (Neosensors, Durham, UK, CUV991200).
2. 500 mL of 20 mM HEPES-KOH, 138 mM NaCl, 2.7 mM KCl, 2 mM EDTA, pH 7.4 plus 0.05% Tween-20. (HBSTE).

3. 100 µL of 5 mg/mL avidin (Pierce, 21121) dissolved in high-purity water. Streptavidin and NeutrAvidin can be used instead of avidin.
4. 250 µL of 50 mM *N*-sulfosuccinimidyl 6-(3′-(2-pyridyldithio)-propionamido) hexanoate (Sulfo-LC-SPDP) (Pierce, 21650) dissolved in water.
5. 5 mL of 10 mM sodium acetate pH 5.0.
6. 25 mL of 100 mM HCl.
7. 0.5 mL of 5 mM D-biotin dissolved in HBSTE.
8. 5 mM dithiothreitol (DTT) in water (for amine coupling option).
9. 1 M ethanolamine, pH 8.5.
10. 0.2 mg/mL IgG was used as example. Storage buffer must be free of reducing agents, Tris, and azide.

2.10. Materials for Formation of a Lipid Monolayer on Hydrophobic Surface

1. Hydrophobic chip ((Neosensors, Durham, UK, CUV991500).
2. 500 mL of phosphate buffered saline (PBS) containing 0.025% (w/v) sodium azide and 1 mM EDTA (PBS/AE) pH 7.4. (Sigma, P-3813).
3. 25 mL of 0.1 M HCl.
4. 25 mL of 10 mM NaOH.
5. 50 mL of 2-propanol HPLC grade (Aldrich, 27049-0).
6. 200 µL of 20 mg/mL Dioleoylphosphatidylcholine (DOPC) (Sigma, P-7212) in chloroform containing 0.1% (w/w) butylated hydroxytoluene, store at −18 °C or below.
7. 200 µL of 20 mg/mL Bovine serum albumin (BSA) (Sigma, A-7888) in PBS/AE, store at −18 °C.

2.11. Materials for Immobilizing Phospholipids on Non-Derivatized Surface

1. Non-derivatized chip (Neosensors, Durham, UK, CUV991600).
2. 500 mL of phosphate buffered saline (PBS) pH 7.4.
3. 50 mL of PBS + 1.25% OG (PBS/OG).
4. 5 mL of 2 mg/mL PLs in PBS/OG. In this example a 50:50% mix of dimyristoyl-phosphatidylethanolamine (DMPE) and dioleoyl-phosphatidylglycerol (DOPC).

2.12. Materials for Capture of His-Tagged Proteins

1. Carboxylate functionalized chip (Neosensors, Durham, UK, CUV991300).
2. 500 mL of 10 mM HEPES, 0.15 M NaCl, 50 µM EDTA, 0.005% Tween 20, pH 7.4, (HBSTE).
3. 30 mL of 50 mM HEPES buffer pH 8.5 (HEPES).
4. 5 mL of 5 m 00 µM $NiCl_2$ in HBSTE.

5. 30 mL of 1 M ethanolamine pH 8.5.
6. 30 mL of 50 mM phosphate buffer, 10% glycerol, 30 mM imidazole, 300 mM NaCl, pH 6.8, (PGI wash buffer).
7. EDC stock (1.15 g in 15 mL high-purity water), (*see* **Note 2**).
8. NHS stock (200 mg in 15 mL high-purity water), (*see* **Note 2**).
9. 10 mg/mL NTA (Qiagen, 34491) in 380 µL HEPES buffer.
10. His-tagged protein.

2.13. Materials for Capture of FLAG-Tagged Proteins

1. Carboxylate functionalized chip (Neosensors, Durham, UK, CUV991300).
2. 30 mL of 10 mM acetate buffer pH 4.5.
3. 25 mL of 10 mM HCl (regenerant).
4. Anti-FLAG M2® monoclonal antibody (Sigma, F3165).
5. An irrelevant monoclonal antibody as a control.
6. FLAG peptide (Sigma, F3290).
7. FLAG-BAP control protein (Sigma, P7457).
8. 500 mL of 50 mM phospate buffer saline, 0.05% Tween-20, buffer pH 7.4 (PBS/T).
9. 30 mL of 1 M ethanolamine pH 8.5.
10. EDC stock (1.15 g in 15 mL high-purity water), (*see* **Note 2**).
11. NHS stock (200 mg in 15 mL high-purity water), (*see* **Note 2**).

2.14. Materials for Preparation of Colloidal Gold-Protein Complexes

1. 100 mL of 25 mM potassium carbonate.
2. 10 mL of 10% (w/v) BSA adjusted to pH 9 with NaOH, filtered.
3. Protein to complex in salt free solution.
4. 30 nm colloidal gold sol (Biocell, Cardiff, UK).
5. Rotary mixer and microcentrifuge.

2.15. Materials for Determination the Concentration of Theophylline in Buffer and Serum

1. CMD chip (Neosensors, Durham, UK, CUV991100).
2. 500 mL 10 mM phosphate buffered saline (PBS), 2.7 mM KCl, 137 mM NaCl, pH 7.4, 0.05% Tween. PBS/T).
3. EDC stock (1.15 g in 15 mL high-purity water), (*see* **Note 2**).
4. NHS stock (200 mg in 15 mL high-purity water), (*see* **Note 2**).
5. 5 mL 10 mM sodium acetate, pH 4.5.
6. 10 µg/mL Rabbit anti-mouse Fc (RAMFc) were supplied by FAST.
7. Thyophyline-8-butyric acid lactam (Calbiochem) (theophylline-protein-conjugate) were supplied by FAST.

8. 100 µg/mL MAb was from Biogenesis (Bournemouth, UK).
9. Prepare solutions of theophylline (Aldrich, Dorset, UK) in the range 0–40 µg/mL in PBS/T.
10. Mix theophylline solutions with equal volumes of theophylline-protein-conjugate solution (final concentration 200 µg/mL).
11. Prepare solutions containing only conjugate.
12. Prepare solutions of theophylline (Aldrich, Dorset, UK) in the range 0–40 µg/mL in serum and dilute it in 1:10 in PBS/T prior mixing with theophylline-protein-conjugate.

2.16. Materials for Protein–Carbohydrate Recognition

1. CMD chip (Neosensors, Durham, UK, CUV991100).
2. 500 mL 10 mM phosphate buffered saline (PBS), 138 mM NaCl, 2.7 mM KCl, pH 7.4, 0.05% Tween. (PBS/T).
3. 2 M NaCl, 10 mM Na_2HPO_4, pH 7.2. (used as generation buffer between analysis of different samples of the same ligate).
4. 2 M guanidine HCl (Post regeneration when different ligate type was used).
5. EDC stock (1.15 g in 15 mL high-purity water), (*see* **Note 2**).
6. NHS stock (200 mg in 15 mL high-purity water), (*see* **Note 2**).
7. *N*-hydroxysuccinimide amino caproate (LC) biotin (Pierce).
8. Human bFGF was purified as described *(30)*. Different concentrations were prepared (20–330nM).
9. Heparin from porcine intestinal mucosa (Sigma).
10. Streptavidin (Sigma).
11. bFGF peptides (–55 to –43, 52–61, 117–126, and 127–140) were synthesized and purified as described in *(31)*. Different concentrations were prepared (2–93 µM).
12. Amino groups of heparin were biotinylated as described *(31)*.

2.17. Materials for Nucleic Acid Hybridization

1. CMD chip (Neosensors, Durham, UK, CUV991100)
2. 200 µg/mL streptavidin (Sigma) in 10 mM acetate buffer pH 5.0.
3. 20 mM HCl.

Biotinylated oligonucleotides were prepared using biotin phosphoramidite using Applied Biosystem Synthesiser resulting in the biotinylation at the 5′ end.

4. *Oligo-1*. A biotinylated 19mer oligonucleotide with 53% GC content. T_m = 58 °C *(1)*.
5. *Oligo-2*. A 40mer oligonucleotide with 50% GC content, containing at its 3′ end a 19 base sequence complementary to oligo-1.
6. *Oligo-3*. A 50-mer oligonucleotide with no complementary to oligo-1. Approximately 50% GC content.

7. *Oligo-4*. A biotinylated 19-mer oligonucleotide with no complementary to oligo-2. 53% GC content.
8. 500 mL of 10 mM phosphate buffered saline (PBS), 2.7 mM KCl, 137 mM NaCl, pH 7.4, 0.05% Tween, PBS/T).

2.18. Materials for Fermentation Monitoring

1. CMD chip (Neosensors, Durham, UK, CUV991100).
2. 500 mL of 10 mM phosphate buffered saline (PBS), 0.5 M NaCl, 2.7 mM KCl, pH 7.4, 0.05% Tween. (PBS/T).
3. 10 mM acetate buffer pH 5. Acetate buffer was made by titrating sodium acetate (AnalaR grade) with 2 M acetic acid.
4. Hen leg lysozyme (Sigma, L-7001).
5. D1.3 Fv produced by batch fermentation of *E. coli* following a protocol described in *(32)*.

2.19. Materials for Bacterial Cells Detection

1. AS chip (Neosensors, Durham, UK, CUV991400).
2. 500 mL 10 mM phosphate buffered saline (PBS), 137 mM NaCl, 2.7 mM KCl, pH 7.4.
3. *Staphylococcus aureus* strain (Cowan-1) and (Wood 46) (Sigma).
4. Human immunoglobulin IgG (ICN).
5. Bovine serum albumin (BSA) (Sigma).
6. 25% Glutaraldehyde in water (Sigma).
7. Colloidal gold sol (30 nm diameter) (Biocell).
8. Prepare colloidal gold human IgG conjugate as described in *Subheading 6.3.9 (33)*. Dilute the colloidal gold-IgG particles to a constant O.D.530 in PBS buffer.
9. Haemocytometer for determining cell concentrations in stock suspension.

2.20. Materials for Receptor–Cell Interactions

1. AS chip (Neosensors, Durham, UK, CUV991400).
2. *Bis*[sulfosuccinimidyl] suberate (BS^3) (Pierce, 21580).
3. 1 mg/mL BS^3 in PBS (PBS/BS^3).
4. 500 µL 0.1 M HCl.
5. 500 mL 10 mM sodium phosphate buffered saline, pH 7.4, 134 mM NaCl, 3 mM KCl, (PBS).
6. 500 mL 10 mM sodium phosphate buffered saline, pH 7.4, 134 mM NaCl, 3 mM KCl, (PBS/T), 0.05% Tween 20. Tween 20 (Pierce and Wariner, Surfact-Amps® 20, 28320).
7. 100 µg/mL BSA in PBS (Sigma, A-6003).
8. Blocking solution 2 mg/mL BSA in PBS.
9. 130 µg/mL PR3B10 antibody *(34)* in PBS.
10. Cells were grown in DMEM medium with 10% v/v fetal calf serum and re-suspended in PBS/BSA. Murine L cells,

co-transfected with a full length CEA cosmid clone *(35)*, and pSVneo, selected for neomycin resistance, were used as a source of CEA bearing cells.

11. Untransformed cells were used as control.

2.21. Materials for Determination of the Kinetic Constants

1. CMD chip (Neosensors, Durham, UK, CUV991100).
2. 500 mL 10 mM phosphate buffered saline (PBS), 137 mM NaCl, 2.7 mM KCl, pH 7.4, 0.05% (v/v) Tween 20. (PBS/T).
3. *Staphylococcus aureus* protein A (Sigma, P 3838).
4. Prepare different concentrations (2–60 nM) of human immunoglobulin IgG (ICN) in PBS/T.
5. Bovine serum albumin (BSA) (Sigma).
6. 10 mM acetate buffer pH 4.5 was prepared by titrating sodium acetate (AnalaR grade) with 2 M acetic acid.
7. EDC stock (1.15 g in 15 mL high-purity water), (*see* **Note 2**).
8. NHS stock (200 mg in 15 mL high-purity water), (*see* **Note 2**).

3. Methods

3.1. RM Sensors Chip Fabrication

The resonant mirror sensor chips were fabricated on 1 mm thick Schott SF10 glass substrates, optically polished on both sides (Gooch and Housego Ltd, Illminster, UK). The substrates were cleaned successively in Decon-90 solution and acetone, then dried at 80 °C for 30 min. Deposition of silica spacer layers and titania–hafnia waveguide layers was performed using e-beam assisted vacuum deposition. The sensor substrates were cut into rectangles 10 × 12.5 mm by scribing and fracturing after deposition of the spacer and waveguide layers.

3.2. RM Setup Instrumentation

Figure 2a, b show a schematic of the optical arrangement used for the angular and wavelength scan instrumental configurations. The same optical arrangement used has been detailed elsewhere in more detail *(1, 2, 19–26)*. For the angular scan instruments, a monochromatic light source, such as 670 nm laser diodes or high-intensity red light emitting diodes with an interference filter (*see* **Note 3**), was used and a low power (20 W) tungsten–halogen lamp with suitable filters for the wavelength scan (*see* **Note 4**). On the input side, a light source (LED or halogen–tungsten lamb), with some collimating lens (plano-convex with 25 mm diameter, EFL: 60 mm) to provide a 'clean' beam of light and 350 µm pinholes (53 mm outer diameter and 1.2 mm minimum aperture) was used. This was passed through an optional filter (*see* **Note 5**),

then an input-polarizer with 25 mm diameter, oriented to provide equal TE and TM intensities. In the angular scan mode, the input beam is then passed through a plano-convex cylindrical lens with 25 mm diameter, EFL 100 mm to provide a converging wedge beam, whereas in the wavelength scan mode, the collimated beam is unmodified.

The incoming beam then passes into a high-index (SF10) prism (n = 1.72, Comar instruments, Cambridge, UK) and into the sensor substrate via an index-matching fluid (Cargille Labs Refractive Index Liquid Series RI = 1.730). On the output side, the beam is passed through a quarter-wavelength plate, which acts to remove any additional 'background' phase shift between TE and TM modes. The beam then passes through the output polarizer with 25 mm diameter, which is oriented at about 90° to the input polarizer, to remove all but the light, which has undergone resonance. For the angular scan instrument, this then results in a substantially parallel beam of light covering a narrow range of angles, which can be focused on to a detector using a convex lens (25 mm diameter, EFL:60 mm). As the surface conditions change, the output angle changes and hence the position of the peak on the detector changes. The wavelength scan instrument is more complex. The output beam is still well collimated, but only contains a narrow range of wavelengths, corresponding to those wavelengths that undergo resonance at the fixed input angle. The output beam is allowed to fall on to a grating (a 1,200 lines/mm replicated ruled grating (Ealing Electro-Optics (Watford, Hertfordshire, UK), and the resulting diffracted beam is then focused on to a detector. In both instances, as the surface refractive index changes, the position of the peak on the detector changes.

Two kinds of charged coupled device (CCD) detectors were employed. For the angular scan, a linear CCD detector was used to monitor the device output, consisting of a 5,000-pixel CCD (Sony ILX506A, pixel pitch 7 μm). The CCD output was digitized to a 12-bit resolution and collected via the parallel port of a host computer with software written in-house using C++. For the wavelength scan, a 2-D CCD detector with associated image processing functions was used, based on a Sony ICX039AL CCIR standard monochrome camera chip, resolution 752 horizontal pixels by 582 lines. The output from the CCD sensor was digitized to 8-bit resolution using a flash analogue-to-digital converter at 14.1875 MHz, and passed to a hard-wired signal averager, which allowed up to 256 successive fields to be averaged together to improve the signal-to-noise ratio of the input. The pixel data were stored in 1 MB of fast (35ns) static random access memory (RAM), which could also be accessed by the programmable image processing system. This was based on an Inmos 20 MHz T805 transputer, with 4 MB of program RAM and a high-speed serial link to a host computer. The transputer-based

image processing system eliminated the need for large amounts of data to be transferred to the PC for processing.

3.3. Carboxy Methyl Dextran

This is a three dimensional matrix of 200 nm thick immobilized carboxymethylated dextran on the sensor surface. The dextran is a polymer of glucose residues with a low degree of branching, low polydispersity and average weight of approximately MW = 500,000. The dextran is carboxylated to give approximately one carboxyl group (pK_a = 3.5) per two glucose residues. Hence, the Carboxy Methyl Dextran (CMD) surface is negatively charged in typical buffers used that have a pH > 5. If the pH of the buffer is less than the isoelectric point (PI) of a protein, the protein will exhibit a net positive charge so it will promote its electrostatic pre-concentration into the dextran prior to covalent immobilization. The CMD enables simple coupling of amino-containing molecules to the carboxylate group of the matrix through succinimidyl ester chemistry resulting in the formation of a peptide bond between the amino-containing molecules and the matrix (**Fig. 3a**). The advantage of the CMD coated surface is that the 3-D nature of the hydrophilic matrix increases the ligand immobilization loading of the system compared to a planar layer. In addition, it keeps immobilized ligands away from direct contact with the surface, resulting in greater retention of the biological activity of biomolecules, reducing protein denaturation and non-specific adsorption. The disadvantage of this surface is that the orientation of immobilized recognition molecules will not be uniform due to the random nature of the immobilization chemistry. The second disadvantage is that this matrix can only be used for small analytes as the large analytes such as cells may be size-excluded. Thirdly, the molecule's binding will not be sensed equally in the matrix due to the exponential nature of the evanescent field. In addition, from the kinetics point of view, molecules bound to sites farthest from the surface may sterically hinder the binding of other molecules to sites within the matrix *(36, 37)*.

3.3.1. Procedure for Dextran Coating

1. Silanized chips are immersed overnight in 23% (w/w) dextran solution in 0.1 M NaOH (*see* **Note 1**).
2. The following day the chip is washed extensively with water to remove excess dextran.
3. The chip is treated with 1 M bromoacetic acid in 2 M NaOH for 6 h.
4. Rinse the chip extensively with water and dry; then it is ready to use.

3.3.2. Procedure for the Immobilization of Amine-Containing Ligand to CMD

This protocol describes the immobilization of the protein on a dextran coated cuvette in the IAsys instrument.

1. Insert a CMD cuvette into the instrument and wash with PBST for 10 min for equilibration.

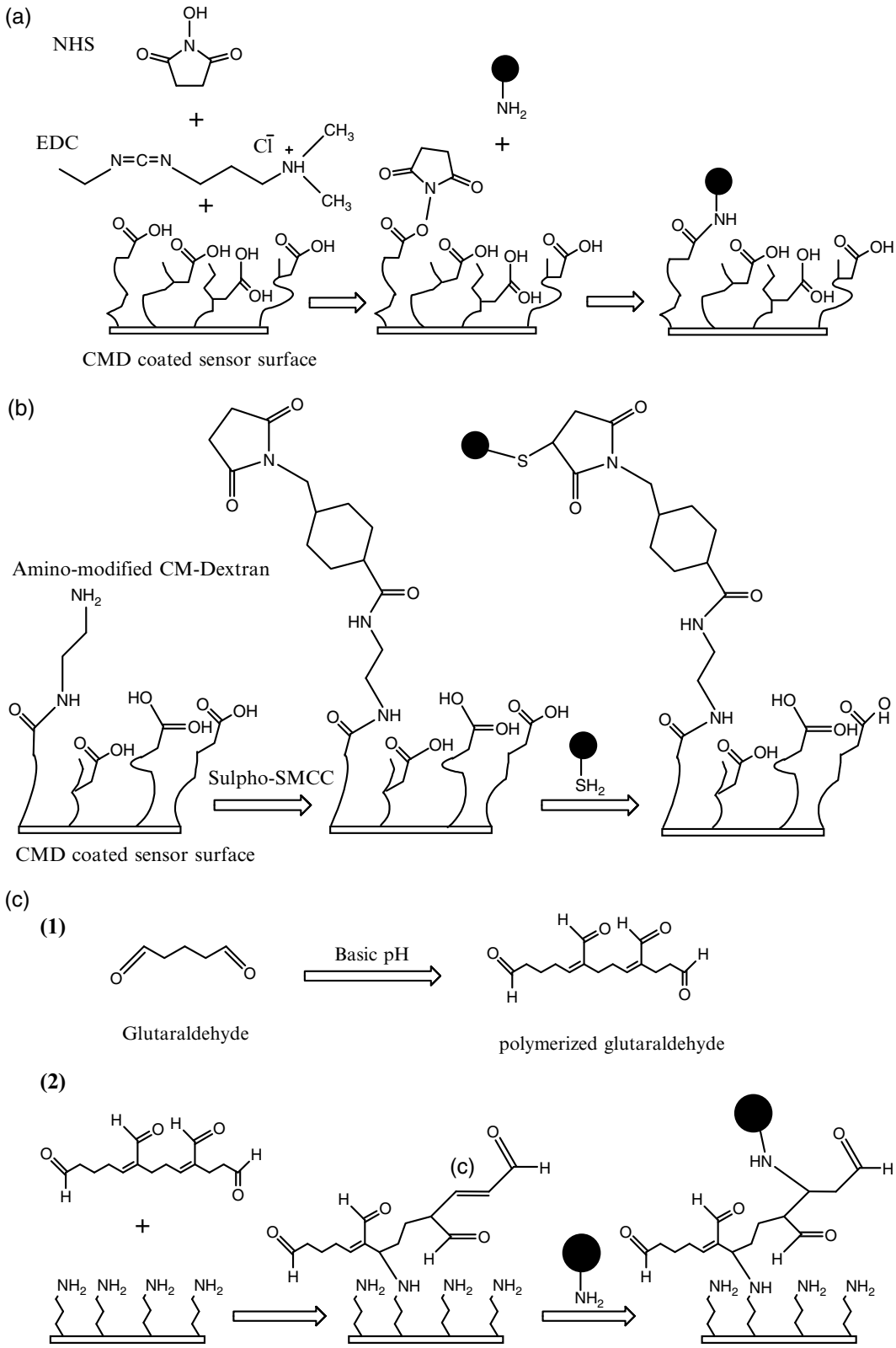
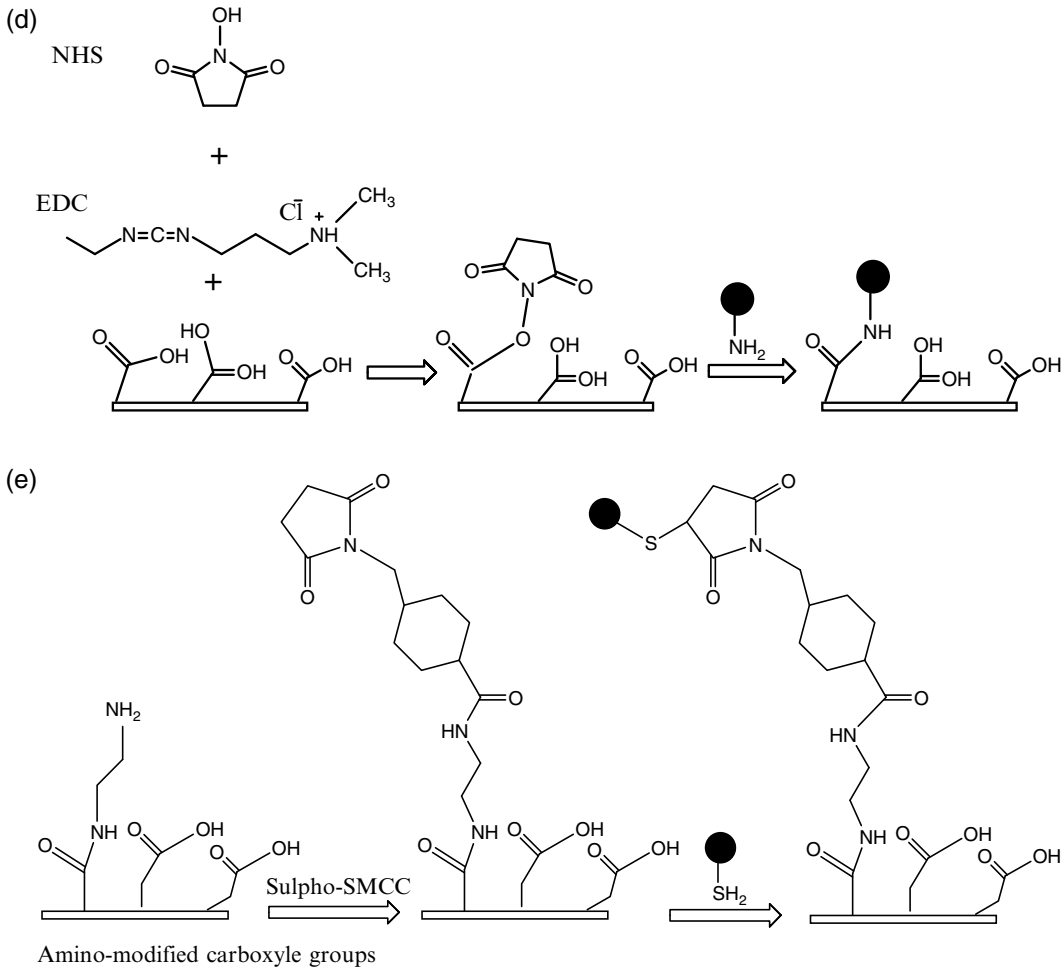


Fig. 3. (**a**) Reaction scheme for carboxymethyl dextran with EDC/NHS for the immobilization of amine containing ligands. (**b**) Reaction scheme for Sulpho-SMCC modified immobilization of thiol containing ligand. (**c**) Reaction scheme (A) polymerization of glutaraldehyde and (B) the cross-linking of a ligand to an amino modified sensor surface.



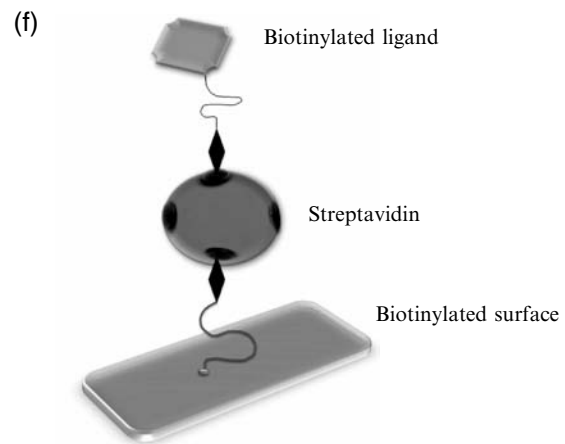


Fig. 3. (continued) (**d**) Reaction scheme for carboxylate surface with EDC/NHS for the immobilization of amine containing ligands. (**e**) Reaction scheme for sulpho-SMCC mediated immobilization of thiol containing ligands. (**f**) Schematic diagram of capturing biotinylated ligand onto biotin functionalized surface via streptavidin.

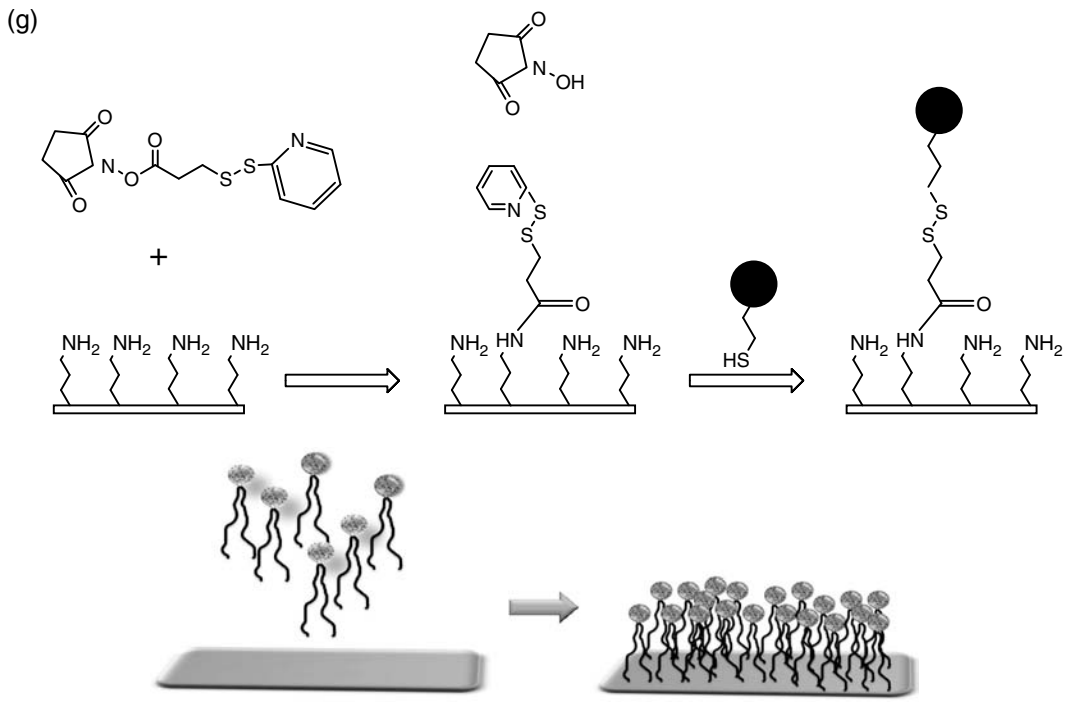


Fig. 3. (continued) (**g**) Schematic reaction diagram of surface amines with SPDP, and subsequent reaction with free, accessible thiol from protein. (**h**) Formation of a lipid monolayer from solution onto hydrophobic sensor surface.

2. Start data acquisition and collect baseline data for 3 min.
3. Introduce a mixture of 200 µL of EDC and 200 µL of NHS for 7 min (*see* **Notes 2** and **6**).
4. Wash with PBST and collect baseline data for 3 min (*see* **Note 7**).
5. Change the PBST to 10 mM acetate buffer pH 5.5 to get a baseline (*see* **Note 8**).
6. Introduce IgG in acetate buffer to the cuvette for 5 min to begin protein electrostatic uptake prior covalent binding.
7. Wash with PBST and collect baseline data for 3 min.
8. Block the unreacted NHS-ester with 1 M ethanolamine pH 8.5 for 3 min (*see* **Note 9**).
9. Wash with PBST to get the baseline.
10. Wash the cuvette with 10 mM HCl for 3 min to remove the non-covalently bound ligand to the matrix.
11. Wash with PBST and collect baseline data for 3 min.
12. Calculate the amount of immobilized protein by subtracting the baseline level at **step 4** from that at **step 11**.

3.3.3. Procedure for the Immobilization of Thiol-Containing Ligand to CMD

This protocol describes a method for immobilization of a thiol containing ligand to CMD coated sensor surface as shown in **Fig. 3b**. This approach will include the modification of the CMD with a primary amine, followed by reaction with an amine–thiol reactive heterobifunctional cross-linking agent Sulfo-succinimide 4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate (Sulfo-SMCC). Covalent immobilization is achieved by the direct addition of thiol-bearing ligands to the modified sensor surface. Thiol groups offer a good approach to achieving orientation of a ligand, especially with cysteine incorporation into one terminal of a peptide.

Protocol

1. Insert CMD cuvette into the instrument and wash with 200 µL of PBST and allow 10 min for equilibration.
2. Start data acquisition and collect 3 min of baseline data.
3. Incubate the surface with 50 µL of a mixture of 1:1 (v/v) EDC/ NHS for 10 min (*see* **Notes 2** and **6**).
4. Wash with 200 µL of PBST and collect baseline data for 3 min.
5. Incubate the surface again twice with 50 µL of a mixture of 1:1 (v/v) EDC/ NHS for 7 min.
6. Wash with 200 µL of PBST and collect baseline data for 3 min.
7. Incubate the surface with 50 µL of 1 M ethylene diamine pH 8.5 for 10 min to modify the carboxymethyl groups with amines.
8. Wash with 200 µL of PBST and collect baseline data for 2 min.
9. Activate the amine group with Sulfo-SMCC by washing and incubating the surface with 200 µL of 1 mg/mL Sulfo-SMCC for 10 min.
10. Wash with 200 µL of PBST and collect baseline data for 2 min.
11. Incubate the surface with 50 µL of Cys-GM-CSF to covalent binding for 8 min.
12. Wash with 200 µL of PBST and collect baseline data for 2 min.
13. Calculate the amount of immobilized protein by subtracting the baseline level after **step 10** from that after **step 12**.

3.4. Aminosilane Functionalized Surface

The biosensor surface is coated with an aminosilane (AS) derivative, resulting in the introduction of amino groups to the surface via an alkyl chain linker. The amino derivatized surfaces are particularly useful for immobilization of ligands with PI < 4, where methodologies based on electrostatic concentration into a CMD matrix are not effective. The advantage of this chemistry is that

it allows large ligates such as membrane fragments, liposomes, viruses and cells which are unable to penetrate the 3D-hydrogel matrix to enter the most sensitive area of the evanescent field. Highly charged molecules can be used with this surface, which might be electrostatically repelled from the CMD matrix. This surface can be activated with cross-linkers such as glutaraldehyde or bis(sulfosuccinimidyl)suberate. The disadvantage of this method is that there is very little electrostatic concentration of ligand to the surface unlike the charged CMD matrix. This requires higher concentrations of ligand to give satisfactory surface coverage. Typically, concentrations in the range of 0.1–1 mg/mL are required. This protocol will describe immobilization of ligands via their amino groups to the surface amino-functionalized groups using polymerized glutaraldehyde **(Fig. 3c)**.

3.4.1. Procedure for Polymerizing the Glutaraldehyde

1. Polymerized glutaraldehyde (PG) is prepared by adding 500 µL of 0.1 M NaOH to 5 mL of 5% (v/v) glutaraldehyde as shown in **Fig. 3c**(A).
2. Leave for 30 min to polymerize before neutralizing with 500 µL 0.1 M HCl. Presence of PG can be determined from a wavelength scan between 200 and 350 nm. An absorption peak is seen around 234 nm, which represents the polymer, whereas a peak at 280 nm is the monomer. The resulting polymerized glutaraldehyde solution must be stored at or below –18 °C.

3.4.2. Immobilization Procedure

1. Insert new AS cuvette into the instrument and wash with PBS for 10 min for equilibration (*see* **Note 10**).
2. Start data acquisition and collect 3 min of baseline data.
3. Introduce PG to the cuvette for 30 min.
4. Wash with PBS to get stable baseline.
5. Introduce the ligand solution 100 µg/mL and leave it for about 30 min (*see* **Note 11**).
6. Wash the cuvette with 1 M formic acid for 2 min to remove the non-covalently bound ligand to the surface.
7. Wash with PBS for about 5 min to get the baseline.
8. Wash the cuvette with 1 mg/mL BSA for 5 min to block unreacted PG (*see* **Note 12**).
9. Wash with PBS to get the baseline.
10. Calculate the amount of immobilized protein by subtracting the baseline level after **step 4** from that after **step 9**.

3.5. Carboxylate Surface

The planar carboxylate surface allows the user to analyze biomolecular interactions in the absence of the 3-D CMD hydrogel layer. This surface is useful for large molecules (> 1,000 KDa),

particles or cells binding to the recognition molecules closer to the surface, where the evanescent field is more intense. Amino and thiol containing ligands can be immobilized on the carboxylic acid functionalized surface.

3.5.1. Immobilization of Amine-Containing Ligand to Carboxylate Surface

The biosensor surface contains carboxylic acid functional groups that can react with the primary amine of the ligand via amide bond using EDC/NHS chemistry **(Fig. 3d)** as described in **Subheading 3.13.1**.

Protocol

1. Insert carboxylate cuvette into the instrument and wash with PBS for 10 min for equilibration.
2. Start data acquisition and collect 3 min of baseline data.
3. Introduce a mixture of 200 µL of EDC and 200 µL of NHS for 7 min (*see* **Notes 2** and **6**).
4. Wash with PBST to get stable baseline.
5. Change the PBST to 10 mM acetate buffer pH 5.5 to get stable baseline (*see* **Note 8**).
6. Introduce RAMFc in acetate buffer to the cuvette for 10 min to begin electrostatic uptake of protein prior covalent coupling.
7. Wash with PBS for 3 min to get stable baseline (*see* **Note 11**).
8. Block un-reacted NHS-ester and uncovered surface with 2 mg/mL β-casein for 3 min.
9. Wash with PBS and collect 3 min of baseline data (*see* **Note 12**).
10. Wash residual non-covalently bound ligand with 1 M formic acid for 2 min.
11. Wash with PBS for 3 min to get the baseline.
12. Calculate the amount of immobilized protein by subtracting the baseline level after **step 4** from that after **step 11**.

3.5.2. Immobilization of Thiol-Containing Ligand to Carboxylate Surface

This protocol describes a method for immobilization of a thiol containing ligand to carboxylate functionalized sensor surface as shown in **Fig. 3e**. This approach includes modification of the carboxylate modified surface with a primary amine, followed by a reaction with an amine–thiol reactive heterbifunctional cross-linking agent Sulfosuccinimide 4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate (Sulfo-SMCC). Covalent immobilization is achieved by the direct addition of thiol-bearing ligands to the modified sensor surface. Thiol groups offer a good approach to achieving orientation of a ligand, especially with cysteine incorporation into one terminal of a peptide.

Protocol

1. Insert new carboxylate cuvette into the instrument and wash with 200 µL of PBS and allow 10 min for equilibration.
2. Start data acquisition and collect 3 min of baseline data.

3. Wash and incubate the surface with 50 µL of a mixture of 1:1 (v/v) EDC/ NHS for 10 min (*see* **Notes 2** and **6**).
4. Wash with 200 µL of PBS and collect baseline data for 3 min.
5. Wash twice again and incubate the surface with 50 µL of a mixture of 1:1 (v/v) EDC/ NHS for 7 min.
6. Wash with 200 µL of PBST and collect baseline data for 3 min.
7. The activated carboxyl group is then converted to amines by washing and the surface is incubated with 50 µL of 1 M ethylene diamine pH 8.5 for 10 min.
8. Wash with 200 µL of PBS and collect baseline data for 5 min.
9. Block any remaining activated carboxyl groups by washing twice and incubating the cuvette with 200 µL of 1 M Tris pH 8.5 for 5 min.
10. Wash with 200 µL of PBS and collect baseline data for 5 min.
11. Wash extensively with 200 µL of PBST and collect baseline data for 5 min.
12. Activate the amine group with Sulfo-SMCC by washing and incubating the surface with 200 µL of 1 mg/mL Sulfo-SMCC for 10 min.
13. Wash with 200 µL of PBST and collect baseline data for 2 min.
14. Incubate the surface with 50 µL of Cys-GM-CSF to covalent binding for 10 min.
15. Wash with 200 µL of PBST and collect baseline data for 2 min.
16. Calculate the amount of immobilized protein by subtracting the baseline level after **step 13** from that after **step 15**.

3.6. Biotin Surface

This method is widely used for immobilization of oligonucleotides, DNA, antibodies, peptides and other proteins. Usually, in this process biotinylated biomolecules are attached to the biotin functionalized sensor surface by depositing avidin or streptavidin or NeutrAvidin before introducing the biotinylated biomolecules (**Fig. 3f**). The advantage of this method is the convenient and rapid immobilization of biotinylated molecules. Since the interaction between the avidin and biotin is non-covalent, the surface can be regenerated by completely removing the avidin-captured molecules. Two examples will be described here, of immobilizing biotinylated protein ligands (**Subheading 3.6.2**), and biotinylated DNA (**Subheading 3.15**) onto a biotin-functionalized surface using streptavidin.

3.6.1. Immobilization Procedure of Streptavidin

1. Insert new biotin cuvette into the instrument and wash with PBST for 10 min for equilibration (*see* **Note 13**).

2. Start data acquisition and collect baseline data for 3 min.
3. Introduce avidin in PBST and allow the binding to occur for 10 min (*see* **Note 14**).
4. Wash with PBST to get a baseline (*see* **Note 15**).
5. Calculate the amount of immobilized avidin by subtracting the baseline level after s**tep 2** from that after **step 4**.

3.6.2. Immobilization Procedure for Biotinylated Ligand

The following steps begin after the streptavidin coating and washing steps as described in **Subheading 3.6.1**.

6. Introduce biotinylated protein G in PBST and allow the binding to occur for about 5 min.
7. Wash with PBST for 3 min to establish a baseline.
8. Introduce IgG in PBST and allow binding to occur for about 5 min.
9. Wash with PBST for 5 min to get a baseline.
10. Calculate the amount of immobilized protein G by subtracting the baseline level after **step 5** from that after **step 7**.

3.6.3. Immobilization of Thiol Containing Ligands to Avidin

This protocol describes a method for the immobilization of proteins through available surface thiol groups using an amine-to-thiol heterobifunctional linker to the amine groups in an immobilized avidin protein layer (**Fig. 3g**). The remaining carboxylates on the avidin layer then serves to electrostatically attract a protein ligand. Covalent coupling through its free thiol groups then follows. The use of this protein layer in the immobilization of biomolecules allows ligands of interest to be coupled through a variety of cross-linking chemistries. The advantage of using this protein layer is that it is suitable for the immobilization of biomolecules of all sizes and reduces the non-specific binding.

Protein Preparation

1. Dissolve 500 µL of 0.5 mg/mL IgG in HBSTE.
2. Reduce the IgG protein to have free thiol by addition of DTT to a final concentration of 10 mM, and room temperature incubation for 30 min.
3. Desalt the product using a D-salt™ column (Pierce, 43230) using HBSTE as the pre-equilibrium and running buffer.

Desalting Protein

1. Equilibrate the column with 200 mL of HBSTE at room temperature, then add 500 µL of IgG mixture to the column head.
2. After loading, fill the funnel with HBSTE. Collect a number of fractions of drops (260 µL) each; the protein will be present in fraction numbers 6–9.
3. Wash the columns with 150 mL of HBSTE to remove low-molecular weight compounds prior to re-use.

Immobilization Procedure

1. nsert new biotin cuvette into the instrument and wash with 200 µL of HBSTE and allow 10 min for equilibration.
2. Start data acquisition and collect 3 min of baseline data.
3. Introduce 40 µL of avidin in HBSTE in the cuvette and allow binding to occur for about 3 min.
4. Wash with 200 µL of HBSTE and allow excess avidin to dissociate for about 3 min.
5. Wash extensively with 200 µL of 100 mM HCl for 2 min followed by washing with 150 µL of HBSTE for 3 min.
6. Add 60 µL of SPDP in HBSTE in the cuvette and allow the reaction to continue for 10 min.
7. Wash with 200 µL of HBSTE and collect baseline data for 3 min.
8. Wash with 200 µL of 10 mM acetate buffer pH 5.5, and establish baseline data for about 3 min.
9. Introduce 20 µL of protein containing free thiol (reduced IgG in this example) and incubate it for 15 min or desired level.
10. Wash with 200 µL of HBSE and collect baseline data for 3 min.
11. Wash with 200 µL of 100 mM HCl and incubate for 2 min to remove excess ligand.
12. Wash with 200 µL of HBSTE and collect baseline data for 3 min.
13. Add 600 ng/µL of a specific antibody to detect the level of binding to check if the immobilized protein is still active towards its cognate binding partner.
14. Then wash the free antibody from the cuvette and allow it to dissociate for 10 min.
15. Treat the surface with 100 mM HCl for 2 min to remove the bound antibody.
16. Wash and refill the cuvette with HBSTE to reveal a new baseline which matches that at **step 14**.

3.6.4. Immobilization of Amine Containing Ligands to Avidin

This protocol describes a method for the immobilization of proteins through available surface amino groups using an amine-to-thiol heterobifunctional linker to the amine groups in an immobilized avidin protein layer. The remaining carboxyls on the avidin layer then serve to electrostatically attract a protein ligand. Depending on the mode of activation, covalent coupling may then proceed through thiol groups as described in **Subheading 3.6.3**, or through the amine as described in this protocol. As previously mentioned, the use of a protein layer in the immobilization of biomolecules allows ligands of interest of all sizes to be coupled through a variety of cross-linking chemistries and reduces the non-specific binding.

Protocol

1. Insert new biotin cuvette into the instrument and wash with 200 µL of HBSTE and allow 10 min for equilibration.
2. Start data acquisition and collect 3 min of baseline data.
3. Introduce 40 µL of avidin in HBSTE in the cuvette and allow binding to occur for about 3 min.
4. Wash with 200 µL of HBSTE and incubate to allow excess avidin to dissociate for about 3 min. Wash again with 200 µL of HBSTE to establish a baseline.
5. Block biotin binding sites by addition of 20 µL of 5 mM biotin for 1 min.
6. Wash extensively with 200 µL of 100 mM HCl for 2 min followed by washing with 150 µL of HBSTE for 3 min and incubate for 3 min to allow excess avidin to dissociate. Wash again with 200 µL of HBSTE to establish a baseline.
7. Add 60 µL of SPDP in HBSTE in the cuvette and allow the reaction to continue for 10 min.
8. Wash with 200 µL of HBSTE and collect baseline data for 3 min.
9. Wash with 200 µL of 5 mM DTT, and allow thiol reduction to proceed for 7 min.
10. Wash with 200 µL of HBSTE and collect baseline data for 3 min.
11. Repeat the addition of 200 µL of SPDP and allow the activation to continue for 10 min.
12. Wash with 200 µL of HBSTE and collect baseline data for 3 min. This baseline will be used as starting place for the calculation of net immobilized protein.
13. Wash with 200 µL of 10 mM acetate buffer pH 5.5, and establish baseline data for about 3 min.
14. Introduce 20 µL of protein (1 µg IgG as example for electrostatic uptake), and allow reaction to occur for about 15 min or desired level.
15. Wash with 200 µL of HBSE and collect baseline data for 3 min.
16. Wash with 200 µL of 100 mM HCl and incubate for 2 min to remove excess ligand. (Optional step as might be the protein will not withstand this treatment).
17. Wash with 200 µL of HBSTE and collect baseline data for 3 min.
18. Wash with 200 µL of 1 M ethanolamine, pH 8.5 for 3 min to block residual NHS esters.
19. Wash with 200 µL of HBSTE and establish baseline data for about 3 min.

20. Add 100 ng/μL of a specific antibody to detect the level of binding to check if the immobilized protein still active towards its cognate binding partner.
21. Then wash the free antibody from the cuvette and allow to dissociate for 5 min.
22. Treat the surface with 100 mM HCl for 2 min to remove the bound antibody.
23. Wash and refill the cuvette with HBSTE to reveal a new baseline.

3.7. Immobilization of Phospholipids on Hydrophobic Surface

The RM hydrophobic cuvette provides a substrate onto which hydrophobic molecules can be easily deposited. Lipids can be simply deposited from a solution avoiding the preparation of liposomes (**Fig. 3h**). This surface is used for the immobilization of lipids from solutions, obviating the requirement for liposomes *(38)*. The surface is simply exposed to the required mixture of lipids in the solvent, the solvent is diluted out by the addition of buffer, and the lipids are forced to assemble on the surface.

Before starting this protocol, it is essential to clean the IAsys instrument with ultra pure water, and aqueous solution containing 1 mM NaOH, 1% (w/w) SDS, 1% Tween 20, followed by extensive washing with water. To obtain reproducible results it is essential that the lipids used are not oxidized. It is recommended that stock lipid solutions contain butylated hydroxytoluene 0.1% (w/v) and are stored at less than −60 °C for no longer than 2 weeks.

Protocol

1. Insert a new hydrophobic cuvette into the instrument and wash extensively with 250 μL of 2-propoanol.
2. Start data acquisition and collect 3 min of baseline data.
3. Wash extensively with 200 μL of PBS/AE and collect data for about 10 min (*see* **Note 16**).
4. Wash with 300 μL of 2-propanol and collect data for about 3 min.
5. Add 30 μL of DOPC and leave for 2 min (*see* **Note 17**).
6. Wash rapidly with 320 μL of PBS/AE and collect data for about 6 min (*see* **Note 18**).
7. Wash with 250 μL of 0.1 M HCl for 5 min.
8. Wash with 350 μL of PBS/AE and collect data for about 5 min.
9. Wash with 250 μL of NaOH and leave for 1 min.
10. Wash with 370 μL of PBS/AE and collect data for about 5 min. Measure the amount of lipid on the surface (*see* **Note 19**).
11. Add BSA solution to a final concentration of 1.5 mg/mL.

12. Wash with 350 µL with PBS/AE and leave for 3 min.
13. **Steps 10–11** test for coverage of the sensor surface with lipid. In the presence of a complete lipid monolayer no BSA binding should be apparent after washing with PBS/AE.

It is recommended that if saturated lipids (e.g. DPPC and DSPC) are used a rinse (wash three times with 200 µL) with 5% sucrose (w/w) in water is performed before washing with PBS/AE. Saturated lipid micelles will stick to the surface preventing monolayer formation. The increased density of the sucrose enables the micelles to float to the surface and be washed away.

3.8. Immobilization of Lipids on Non-Derivatized Surface

The non-derivatized cuvette offers an alternative surface to the hydrophobic cuvette for the immobilization of phospholipids (PLS). Single or mixed lipids can be immobilized from the solution in the detergent *n*-octyl β-D-glucopyranoside (OG). The lipid-coated surface can then be used for analysis of protein/lipid interactions, or of lipid-associated proteins with other soluble components.

Protocol

1. Insert a new non-derivatized cuvette into the instrument and wash with 150 µL of PBS and allow 10 min for equilibration.
2. Start data acquisition and collect 3 min of baseline data.
3. Wash with 180 µL of PBS/OG, and collect data for about 3 min.
4. Sonicate the PLs for 10 s in a sonicating cleaning bath and introduce 100 µL to the instrument of the PLS solution till the response is about at a plateau.
5. Add 30 µL of PBS without removing the PLS solution. Leave until the binding response has approached a plateau.
6. Wash three times with 50 µL of PBS.
7. The amount of lipid immobilized can be determined by subtracting the initial baseline level in PBS from the final level after stage 6 (*see* **Note 20**).

3.9. Capture of His-Tagged Proteins

Poly-histidine is a commonly used tag on recombinant proteins. This protocol describes a method for the chelation of His-tagged proteins on a nickel charged surface onto a carboxymethyl dextran coated surface. The immobilization process includes coupling of nitrotriacetic acid (NTA) to the dextran using EDC/NHS and charging the NTA with nickel ions. This complex will then be used to capture His-tagged protein from solutions.

Protocol

1. Insert new carboxylate cuvette into the instrument and wash with 200 µL of HEPES and allow 10 min for equilibration.
2. Start data acquisition and collect 3 min of baseline data.

3. Wash and incubate the surface with 180 µL of a mixture of 1:1 (v/v) EDC/NHS for 15 min.
4. Wash with 200 µL of HEPES and collect baseline data for 3 min (*see* **Note 2**).
5. Incubate 50 µL of NTA solution for 8 min (*see* **Note 21**).
6. Wash with 200 µL of HEPES and collect baseline data for 3 min (*see* **Note 22**).
7. Incubate the cuvette with 1 M ethanolamine pH 8.5 for 8 min to block unreacted NHS-esters (*see* **Notes 9** and **23**).
8. Wash with 200 µL of HEPES and collect baseline data for 5 min.
9. Wash three times and incubate the surface with 200 µL of 500 µM of $NiCl_2$ in HBSTE for 6 min.
10. Wash with 200 µL of HEPES and collect baseline data for 5 min.

Optional

11. Wash three times with 200 µL of PGI wash buffer and leave for 5 min to remove loosely associated metal ions from the residual carboxyl's on the CMD.
12. Wash with 200 µL of HEPES and collect baseline data for 3 min.
13. Spike in the His-tagged sample and allow binding
14. Wash with 200 µL of HEPES and collect baseline data for 3 min. If high concentration of His-tagged material has been used, it is possible that a very distinct dissociation phase will follow. This can take several minutes to settle down. It is possible to speed up the process by washing loosely associated material of the surface using PFI wash buffer (optional).
15. Wash and incubate the surface with 200 µL of PGI for 2 min to clear the his-tag from the surface (*see* **Note 24**).
16. Wash with 200 µL of HEPES and collect baseline data for 3 min.

3.10. Capture of FLAG-Tagged Proteins

FLAG is a commonly used tag on recombinant proteins. The tag consists of eight amino acids in the sequence Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys. This protocol describes a method for immobilization of anti-FLAG antibody to CMD for the capture of FLAG-tagged proteins. Captured recombinants can be easily eluted with competing FLAG peptides at nM concentrations *(43)*.

Protocol

1. Insert new carboxylate cuvette into the instrument and wash with 200 µL of PBS/T and allow 10 min for equilibration.
2. Start data acquisition and collect 3 min of baseline data.
3. Wash and incubate the surface with 180 µL of a mixture of 1:1 (v/v) EDC/ NHS for 10 min.

4. Wash with 200 µL of PBS/T and collect baseline data for 3 min.
5. Replace PBS/T with acetate buffer pH 4.5 and leave to equilibrate for 4 min.
6. Introduce Anti-FLAG antibody between 10 and 20 µg/mL and leave for 5 min. An irrelevant antibody can be used immobilized at the same level as a control.
7. Wash with 200 µL of PBS/T and collect baseline data for 3 min.
8. Incubate the cuvette with 1 M ethanolamine pH 8.5 for 4 min to block unreacted NHS-esters.
9. Wash with 200 µL of PBS/T and collect baseline data for 5 min.
10. Wash extensively with 200 µL of 50 mM HCl and leave to equilibrate for 5 min to remove loosely bonded antibodies from the CMD matrix.
11. Wash with 200 µL of PBS/T and collect baseline data for 3 min.
12. Repeat **steps 9** and **10** twice
13. Introduce 740 nM FLAG-BAP control peptide and incubate it for 5 min.
14. Wash with 200 µL of PBS/T and collect baseline data for 5 min.
15. Wash with 200 µL of 50 mM HCl and leave for 2 min to regenerate the surface.
16. Wash with 200 µL of PBS/T and collect baseline data for 5 min.
17. Repeat **steps 13–16** to assess reproducibility of activity (*see* **Note 25**).

3.11. Sensitivity Enhancement Using Colloidal Gold Complexes

Protein-colloidal conjugate cause a higher refractive index and thickness change per binding event than native protein or other biomolecule. As a result, the colloidal gold nano-particles can be used to enhance the sensitivity of the assays. This protocol describes a general method for the preparation of colloidal gold-protein complexes and its use to improve the detection limit of an assay. Gold complexes can be prepared using the De Mey method. *(33)*. The bioactivity of the colloidal gold complexes can be checked by immunoblotting with the appropriate antibody (Biocell gold conjugates technical information and guidelines for use in electron microscopy, Light Microscopy and Immunoblotting.). The average number of protein molecules per colloidal gold particle can be determined from radioactive studies *(40)*.

Colloidal gold is susceptible to aggregation; therefore all glassware and plasticware should be thoroughly cleaned and if possible, silliconized.

Protocol

1. Adjust the pH of the colloidal gold to 0.5 pH units above the PI of the protein with potassium carbonate.
2. Determine the number of gold particles by measuring the absorbance at λ_{max} or 520 nm (A_{520} = 1 is 1.7×10^{11} particles/mL for 30 nm particles).
3. Add protein stepwise to colloidal gold to give a final molar ratio of 100:1 or 10:1, depending on how many protein molecules are required by gold particles. Typical number of particles will be 1×10^{12} particles/mL final concentration.
4. Add BSA to the solution to final concentration between 0.1 and 0.5% of BSA (w/v) to block any remaining exposed sites on the gold.
5. Leave on a rotary mixer for 15 min.
6. Spin the mixture in a microcentrifuge for 20 min.
7. Carefully remove the clear supernatant leaving loose red sediment.
8. Resuspend the gold complex in fresh BSA solution to remove the more free protein.
9. Repeat **steps 6–8**. A good final volume is 1/10th of the starting volume.
10. It is recommended at this stage to retest the concentration of the colloid as described in **step 2**.
11. Store the product in an air-tight siliconized glass vial at 4 °C until it used for assay enhancement (*see* **Subheading 3.17**).

3.12 Measuring Affinity and Rate Constants

Kinetic information indicates how fast the interactants come together, and how fast the resulting complex breaks down. Kinetic information is given by the rate constants of the forward and reverse reaction as shown in **Eq. 1**. The interaction of the ligate (L) with the immobilized ligand (G) on the sensor surface can be represented as:

$$\mathrm{G} + \mathrm{L} \xrightarrow{K_{on}} \mathrm{GL}.$$

$$\mathrm{G} + \mathrm{L} \underset{k_{diss}}{\overset{k_{ass}}{}} \mathrm{GL}.$$

The rate of complex (GL) formation is = K_{ass} (A)(B)

The rate of the reverse reaction (dissociation) = K_{diss} (AB)

$$K_D = \frac{|\mathrm{G}||\mathrm{L}|}{\mathrm{GL}} = \frac{k_{diss}}{k_{ass}} = \frac{1}{K_A},$$

where K_D and K_A are the affinity constants (dissociation equilibrium constant in units (M) and association equilibrium constant in units (M^{-1}) respectively). The parameters K_{ass} (M^{-1} S^{-1}) and K_{diss} (S^{-1}) refer to the association and dissociation rate constants respectively. By measuring (G), (L), and (GL) at equilibrium, the affinity can be determined. The biosensor gives rise to a signal that directly correlates to the amount of complex (GL) which is being formed. The dissociation equilibrium constant K_D refers to the concentration at which 50% of the binding sites are occupied. K_D and K_A are opposite each other. If K_D is low, the affinity of the interaction is high and in case the K_A is high, the affinity is high.

Upon exposure of the ligate to the ligand, initially the forward reaction predominates and GL is formed. With time, as the concentration of GL increases, the reverse reaction becomes significant. Eventually, at equilibrium, the time taken to reach this depends on the kinetics of the interaction and the concentration of the reactants, and the rate of the forward and reverse reaction, will be the same. GL continues to be formed from G and L, and GL continues to dissociate, and concentrations of GL, G and L remain constant.

$$R_{eq} = \frac{R_{max}[L]}{K_D + [L]}.$$

The classical route to determine equilibrium constants, is measuring the amount of complex GL at equilibrium from a fixed value of ligand. R_{max} is the response when all possible ligand sites are occupied. At each concentration of ligate, a value of the response at equilibrium (R_{eq}) is obtained, equivalent to the amount of complex GL formed at equilibrium. At high concentrations of ligate L compared with the ligand G, the assumption that the concentration of L in solution is constant can be made. This is termed pseudo-first order condition. At high concentrations, R_{eq} is the same as R_{max}. By plotting R_{eq} against the free ligate (L) concentration, the concentration required to saturate 50% of the available ligand sites can be determined; this value is the concentration which give rise to the response of $R_{max}/2$.

Rate of complex (GL) formation:

$$\frac{d[GL]}{dt} = k_{ass}[G][L] - k_{diss}[GL].$$

The concentration of free ligand sites decreases with ligate binding, and thus the concentration of free ligate sites at time t, is equal to the maximum number of sites $(G)_0$ minus the sites bound $(GL)_t$ or $((G)_0-(GL)_t)$;

$$\frac{d[GL]}{dt} = k_{ass}[G]_0 - [GL]_t[L] - k_{diss}[GL]_t.$$

As complex (GL) formation results in a change in response (R_t), which is directly proportional to (GL), R_{max} is equivalent to $(L)_0$ and the above equation can be rewritten as:

$$\frac{dR_t}{dt} = k_{ass}(R_{max} - R_t)[L] - k_{diss}R_t.$$

Multiplying out the brackets and rearranging gives:

$$\frac{dR_t}{dt} = k_{ass}(R_{max}[L] - R_t(K_{ass}[L] + K_{diss}).$$

Originally this led to biosensor data being analyzed by linearization of the data for each ligate concentration (L) by plotting dR/dt against R_t. This should give a straight line of slope k_{ass} [L] + k_{diss}. Plotting slopes against concentrations of L will then give a straight line of slope equal to the association rate constant (K_{ass}), and the intercept is equal to the dissociation rate constant (K_{diss}). However, a problem with linearization is that the errors are compounded. By integration the equation can be transformed into a form suitable for application of non-linear regression:

$$R_t = R_{eq}\left(1 - e^{-(k_{ass}[L]k_{diss})t}\right).$$

Using the above equation, binding data can be fitted at different concentrations to determine K_{on} (where $K_{on} = K_{ass}$ [L] + K_{diss}. A plot of K_{on} against ligate concentration (L), will give a straight line with a slope of K_{ass} and an intercept K_{diss}. The determination of K_{diss} from the intercept of K_{on} vs. (L) often has a high extrapolation error. A more robust approach is to initiate dissociation by removing the ligate with a buffer wash. Under these conditions (i.e. L = 0) dissociation of the ligate from the immobilized ligand is described by **Eq. 3**:

Here the concentration of L is taken to be zero and the first order decay in response is given by:

$$\frac{dR}{dt} = -K_{diss}R_t.$$

On integration it gives:

$$R_t = R_0\ e^{-(k_{diss}t}, \qquad (3)$$

where R_0 is the response at the initiation of dissociation. This simple equation is not applicable to all dissociation profiles due to incomplete dissociation which has be attributed to rebinding of the dissociated ligate.

3.13. Determination of the Concentration of Theophylline in Buffer and Serum

Theophylline has a molecular weight of 180 Da. The response of the RM for the direct detection of any analyte less than 5,000 Da will be too small to be measured reliably. Alternatively, indirect assay format must be employed. The assay is based on competition

between theophylline and a protein-theophylline conjugate for binding to a theophylline monoclonal antibody (Mab) captured on the sensor surface by an immobilized specific rabbit-mouse antibody (RAM-Fc) through the Fc region. The theophylline conjugate used in this assay is IgG (150,000 Da); it contains approximately 20 theophylline residues per protein molecule.

3.13.1 Assay Procedure in PBS/T

1. Insert new CMD cuvette into the instrument and wash with 200 µL of PBS/T and allow 10 min for equilibration.
2. Start data acquisition and collect 3 min of baseline data.
3. Wash and incubate the surface with 180 µL of a mixture of 1:1 (v/v) EDC/ NHS for 10 min.
4. Wash with 200 µL of PBS/T and collect baseline data for 3 min.
5. Introduce 10 µg/mL of RAM Fc for immobilization on CMD using the EDC/NHS chemistry.
6. Wash with 200 µL of PBS/T and collect baseline data for 3 min.
7. Incubate the cuvette with 1 M ethanolamine pH 8.5 for 4 min to block unreacted NHS-esters.
8. Wash with 200 µL of PBS/T and collect baseline data for 5 min.
9. Introduce 100 µg/mL of MAb for 10 min.
10. Wash with PBS/T and collect baseline data for 5 min.
11. Incubate a solution containing one of the theophylline/conjugate mixtures into the cuvette for 10 min.
12. Wash with PBS/T and collect baseline data for 5 min.
13. Regenerate the surface to remove the anti-theophylline and theophylline-protein-conjugate with 50 mM HCl for 3 min, followed by re-equilibration into PBS/T buffer.
14. **Steps 5–13** were then repeated for further theophylline/conjugate mixtures.
15. Construct the calibration curve.

3.13.2 Assay Procedure in Serum

Repeat the same experimental procedure as described for PBS/T. With the exception of theophylline, solutions were prepared in serum and were diluted 1:10 in PBS/T prior to mixing with the theophylline-protein-conjugate. In addition, a control for non-specific binding was performed with a digoxin-protein-conjugate (*see* **Note 26**).

3.14 Protein–Carbohydrate Recognition

The importance of protein–carbohydrate recognition in modulating key cellular activities, such as receptor binding, adhesion, and mitogenesis, has become increasingly recognized in recent

years. Basic fibroblast growth factor (bFGF) is the archetype of the FGF family of growth factors and is involved in the regulation of growth and morphogenetic events as diverse as the induction of mesoderm and limb buds to the growth of skeleton and the mammary gland *(41)*. To elicit their cellular effects, bFGF, like the other FGFs, must interact with a dual receptor system consisting of the receptors tyrosine kinases and heparan sulfate proteoglycans; bFGF binds to the heparin sulfate chains of the proteogylcans *(42)*. Heparan sulphate is a sulphated glycosaminoglycan present on the surface of most mammalian cells. Heparin, a specialized form of heparan sulphate produced by mast cell, contains binding sequences for most heparan sulphate binding proteins, including bFGF *(41)*. A number of peptides derived from the protein sequence of bFGF have been identified that are implicated in heparin binding *(41)*. The RM used to study the interaction of heparin to bFGF was capable of quantifying the very low association rates and affinities of bFGF-derived peptides for heparin. Novel insights on structure and function were therefore obtained. A full report of this work can be found in *(43)*.

Protocol

1. Insert new CMD cuvette into the instrument and wash with 200 µL of PBS/T and allow 10 min for equilibration.
2. Start data acquisition and collect 3 min of baseline data.
3. Deposit streptavidin at a concentration of 200 µg/mL in 10 mM acetate buffer pH 5.0 for 15 min as described in **Subheading 3.6.1**.
4. Wash with 200 µL of 20 mM HCl for 3 min to remove non-covalently attached streptavidin.
5. Wash with 200 µL of PBS/T and collect baseline data for 3 min.
6. Introduce 100 µL of 1 mg/mL of biotinylated heparin and incubate for 30 min.
7. Regenerate the surface with Na_2HPO_4 buffer for 5 min.
8. Wash with PBS/T and collect baseline data for 5 min.
9. For association 100 µL of minimum five different concentrations per ligate (bFGF and peptides) in the PBS/T were analyzed for 5–10 min on at least two different surfaces CMD surfaces and one AS surface.
10. For dissociation run 200 µL of PBS/T for at least 5 min.
11. Regenerate the surface with Na_2HPO_4 buffer for 3 min, followed by re-equilibration in PBS/T.
12. Apply bFGF and bFGF-derived peptides as control analysis onto streptavidin surfaces in the absence of captured heparin (should not show significant interaction).

13. Calculate the kinetic association and dissociation constants (k_{ass} and k_{diss}) and the dissociation equilibrium constant (K_D) as described in **Subheading 3.12**.

Using a series of profiles for bFGF and bFF-peptide binding to heparin to generate the kinetics and equilibrium constants shown in **Table 1** (*see* **Note 27**).

3.15 Nucleic Acid Hybridization

This protocol describes the use of RM for the rapid and unlabeled single stranded DNA–DNA hybridization using a biotinylated oligonucleotide, attached to CMD coated sensor surface via streptavidin *(44)*. This approach is a convenient, generic approach and biotinylated oligonucleotides can be easily synthesized. The binding of biotin to streptavidin is virtually irreversible, requiring extreme conditions to separate the two molecules *(45, 46)*.

Protocol

1. Insert new CMD cuvette into the instrument and wash with 200 µL of PBS/T and allow 10 min for equilibration.
2. Start data acquisition and collect 3 min of baseline data.
3. Streptavidin was immobilized at a concentration of 200 µg/mL streptavidin in 10 mM acetate buffer pH 5.0 for 15 min as described in **Subheading 3.6.1**.
4. Wash with 200 µL of PBS/T and collect baseline data for 3 min.
5. Wash with 200 µL of 20 mM HCl for 3 min to remove non-covalently attached molecules.
6. Wash with 200 µL of PBS/T and collect baseline data for 3 min.

Table 1
kinetic and equilibrium constants for the interaction of bFGF with heparin

	Ligate	
Constant (units)	BFGF (±SE)	BFGF (127–140) (±SE)
K_D (M)[a]	84 (±55) × 10^{-9}	30 (±4) × 10^{-6}
K_D (M)[b]	74 (±20) × 10^{-9}	35 (±7) × 10^{-6}
K_{ass} ($M^{-1}S^{-1}$)[c]	9.3 (±2.5) × 10^{-4}	4 (±0.16) × 10^{-2}
K_{diss} (S^{-1})[d]	6.8 (±0.4) × 10^{-3}	1.4 (±0.38) × 10^{-2}

[a] K_D calculated from five concentrations of ligate in three experiments (two carried out on CM-Dextran and one on AS) by binding curve analysis. K_D was calculated from $K_D = (K_{diss}/K_{ass})$
[c] Mean of three determinations (±SE)
[d] Mean of nine determinations (±SE). (Reproduced from **ref. *48*** with permission from NeoSensors Ltd.)

7. Introduce 2 mg/mL Oilgo-1 in PBS/T and incubated for 15 min.
8. Monitor the binding response after washing with 200 µL of PBS/T and collect baseline data for 3 min.
9. Wash the surface with hybridization buffer (pH 8, 5× saline, sodium citrate, 5× Denhardts solution), followed by 5 mM sodium phosphate, 0.1% v/v Tween 20.
10. Introduce 4 µg/mL complementary oligonucleotides (Oilgo-2) in hybridization buffer and incubate for 15 min.
11. Wash the surface with hybridization buffer and monitor the shift in resonance position **(Fig. 4)** (*see* **Note 28**).
12. Run control experiments on new streptavidin derivatized surfaces using (*see* **Note 28**):
    (a) Oligo-3 (5 µg/mL, 303 nM) with immobilized Oligo-1 at the sensor surface **(Fig. 4)**.
    (b) Oligo-4 (2 µg/mL) with immobilized Oligo-2 (4 µg/mL, 303 nM) at the sensor surface.

Introduce Oligo-2 (4 µg/mL, 303 nM) in the absence of an immobilized specific oligonucelotide.

3.16. Fermentation Monitoring

The fermentation of micro-organisms and mammalian cells for the production of recombinant proteins is an area of increasing industrial interest. It could benefit greatly from the rapid assay to quantify the bioproducts of interest during the fermentation process. Here is one example reported for the use of the RM for

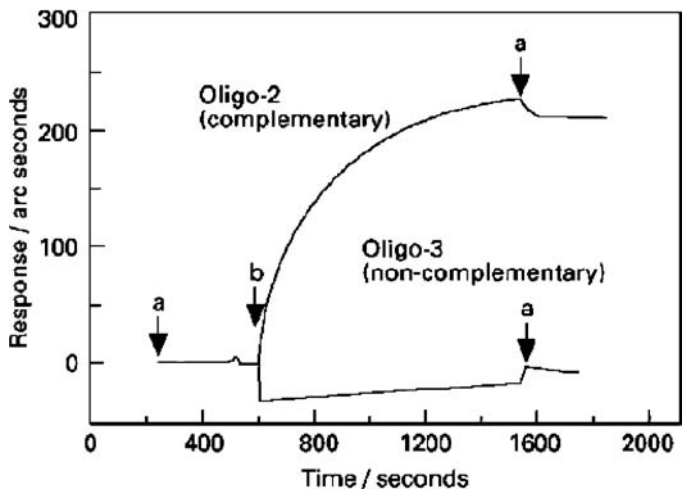


Fig. 4. Specific hybridization of Oligo-2 to captured complementary biotinylated Oligo-1 and the absence of hybridization of non-complementary Oligo-3 with Oligo-1. *Arrows* indicate: (**a**) addition of hybridization buffer, (**b**) addition of oligonucleotides. (Reproduced from **ref.** *46* with permission from NeoSensors Ltd.).

monitoring the recombinant antibody fragment, D1.3 Fv (specific for hen egg lysozyme, HEL) *(47)* produced by periplasmic secretion in *E. coli* fermentation broths *(48)*.

Protocol

1. Insert new CMD cuvette into the instrument and wash with 200 µL of PBS/T and allow 10 min for equilibration.
2. Start data acquisition and collect baseline data for 3 min.
3. Immobilize HEL (30 µg/mL) in acetate buffer pH 5 on CMD matrix for 5 min.
4. Wash with 200 µL of PBS/T and collect baseline data for 3 min.
5. Incubate the surface with 50 µL of 1 M ethylene diamine pH 8.5 for 10 min to modify the carboxymethyl groups with amines.
6. Wash with 200 µL of PBST and collect baseline data for 3 min.
7. Construct calibration curve using (negative) *E. coli* fermentation broth (one does not express D1.3 Fv that mimicked the process broth in terms of operation and final protein concentration *(49)* and by spiking different concentration of purified D1.3 into this broth.
8. Wash with 200 µL of PBS/T and collect baseline data for 3 min.
9. Incubate the sample from the fermenter at regular intervals for 5 min after removing the particulates using microcentrifuge 11,600 × *g* for 5 min.
10. Wash with 200 µL of PBS/T and collect baseline data for 3 min.
11. Regenerate the surface from the immobilized ligand with 100 mM HCl for 4 min.
12. Data can be analyzed using two methods either measuring the absolute change in response after incubating the sample with the ligand for 5 min or measuring the initial binding rate (using linear regression using FASTfit™ software) *(50)*.

3.17. Bacterial Cell Detection

The RM has been used to distinguish between bacterial strains on the basis of their surface proteins. *Staphylococcus aureus* (Cowan-1) cells, which express protein A on their surface, could be detected by binding to Human IgG *(51, 52)*. *Staphylococcus aureus* strain (Wood-46) cells do not interact significantly with Human IgG as they do not express protein A on their surface *(53)* (*see* **Note 29**).

Protocol

1. Insert AS cuvette into the instrument and wash with 200 µL of PBS and allow 10 min for equilibration.

2. Start data acquisition and collect 3 min of baseline data.
3. Activate the surface with 5% (v/v) glutaraldehyde in water for 30 min.
4. Wash with 200 µL of PBS and collect baseline data for 3 min.
5. Incubate the surface with 100 µg/mL IgG in PBS for 8 min.
6. Wash with 200 µL of PBS and collect baseline data for 3 min.
7. Block the surface with 3 mg/mL BSA for 5 min.
8. Wash with 200 µL of PBS and collect baseline data for 3 min.
9. Introduce *S. aureus* (Wood-46) and incubate for 20 min.
10. Wash with 200 µL of PBS and collect baseline data for 3 min (*see* **Fig. 5a** for the results).
11. Repeat the same procedure from **steps 1** to **10** for Wood-46 strain (*see* **Fig. 5a** for the results).
12. Repeat the same procedure from **steps 1** to **10** for *S. aureus* (Cowan-1) on BSA immobilized surface as control surface (*see* **Fig. 5a** for the results).

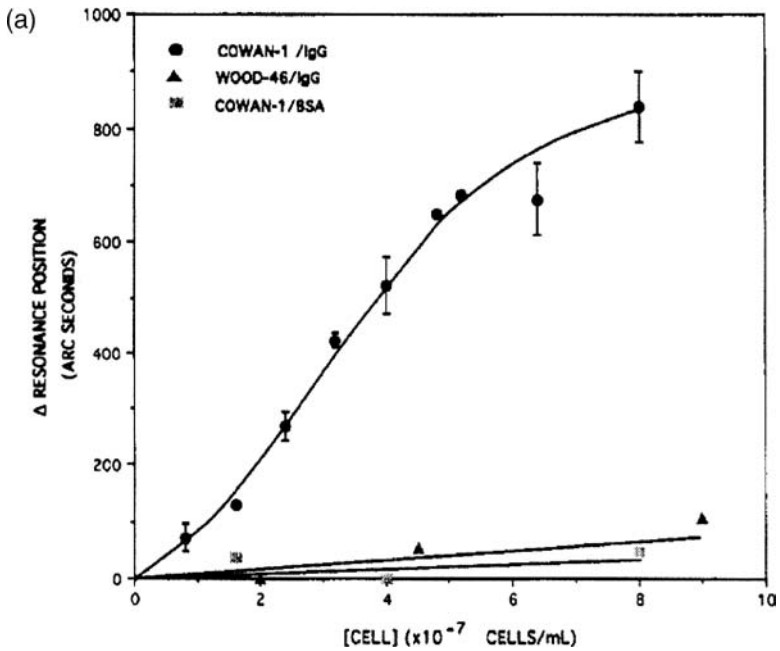


Fig. 5. (**a**) Calibration curve showing the response against cell concentration. The control data for bacterial cell strain Wood-46 and for the interaction between the Cowan-1 and immobilized BSA are also shown. (Reproduced from **ref.** *19* with permission from Analytical Chemistry). (**b**) Comparison of the direct binding assay with the colloidal gold sandwich assay. (Reproduced from **ref.** *19* with permission from Analytical Chemistry).

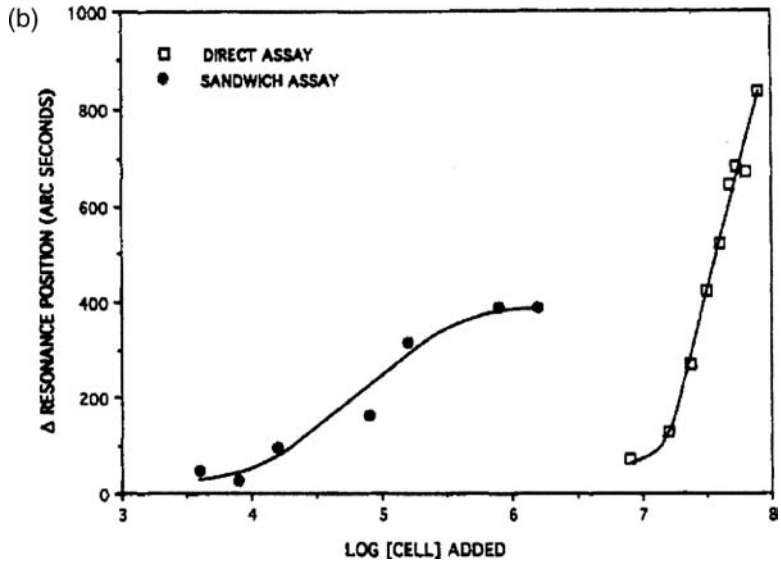


Fig. 5. (continued)

13. For sandwich assay using gold complex: repeat the same procedure as in direct assay from **steps 1** to **10** followed by the addition of colloidal gold human IgG and incubate for 10 min.
14. Wash with 200 µL of PBS and collect baseline data for 3 min (*see* Fig. **5b** for the results).

3.18. Receptor–Cell Interactions

Carcinoembryonic antigen (CEA) *(54)* has been identified as immunocytochemical on tumor cell membrane from a variety of tissues, making it one of the most useful human tumor markers *(55)*. Cell lines have been developed expressing CEA to study the etiology of carcinomas. In the present protocol the RM is used for the real-time detection of the binding of L cells bearing a cell-expressed CEA antigen, to anti-CEA antibody immobilized on AS sensor surface (*see* **Note 29**).

Protocol

1. Insert new AS cuvette into the instrument and wash with PBS for 5 min for equilibration.
2. Start data acquisition and collect 3 min of baseline data.
3. Introduce PBS/BS^3 to the cuvette for 10 min
4. Wash with PBS to get stable baseline.
5. Introduce the antibody solution 130 µg/mL and leave it for about 10 min (*see* **Note 8**).
6. Block remaining activated groups with 2 mg/mL BSA in PBS for 10 min.

7. Wash the cuvette with 20 mM HCl for 2 min
8. Wash with PBS/BSA for about 5 min to get the baseline.
9. Introduce cell solution.
10. Wash with PBS/BSA and collect 5 min of baseline data.
11. Regenerate the surface with 0 mM HCl for 2 min, PBS/T for 2 min, and finally for another 2 min with 20 mM HCl (*see* **Fig. 6**) (*see* **Note 30**).
12. Repeat **steps 2–11** with non-expressing cells (Reference cells) (*see* **Fig. 6**).

3.19. Determination of the Kinetic Constants

The use of RM for measuring equilibrium binding constants is attractive due to the label free detection unlike commonly used techniques such as ELISA, RIA, hapten inhibition, and fluorescence methods *(56)*. This protocol describes the use of RM to determine the rate and affinity constants of the binding interaction between immobilized *Staphylococcus aureus* protein A and different concentration of human IgG in solution *(57, 58)*.

Protocol

1. Insert CMD cuvette into the instrument and wash with 200 µL of PBS and allow 5 min for equilibration.

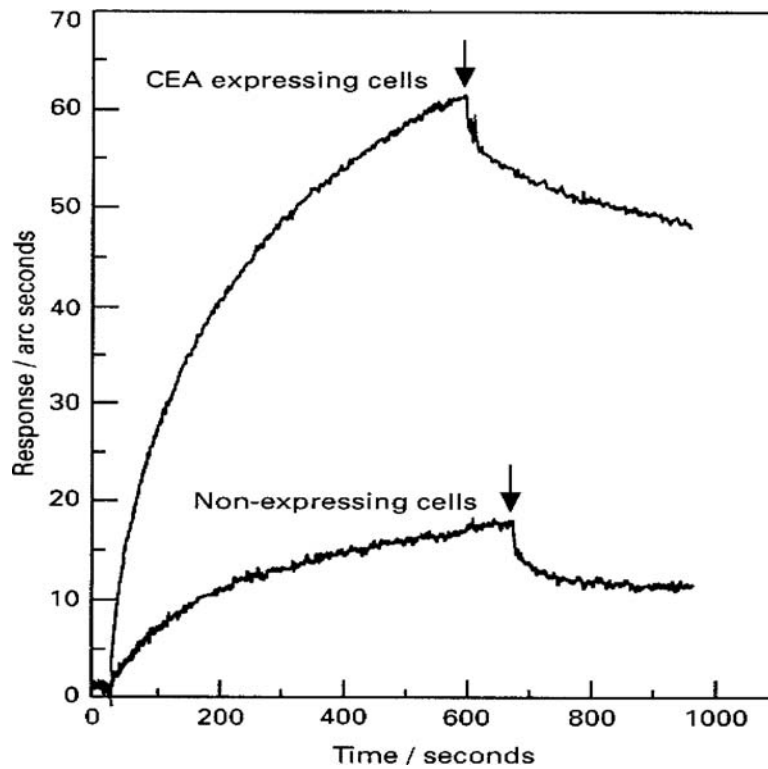


Fig. 6. Binding of L cells to an AS surface derivatized with an anti-CEA antibody. (Reproduced from **ref.** *56* with permission from NeoSensors Ltd.).

2. Start data acquisition and collect 3 min of baseline data.
3. Introduce a mixture of 200 µL of EDC and 200 µL of NHS for 7 min.
4. Wash with PBST and establish a stable baseline.
5. Change the PBST to 10 mM acetate buffer pH 5.5 and collect 3 min baseline data.
6. Introduce 200 µL of 12.5 µg/mL *Staphylococcus aureus* protein in acetate buffer to the cuvette for 15 min.
7. Block the unreacted NHS-ester with 1 M ethanolamine pH 8.5 for 3 min.
8. Wash with PBST to get the baseline.
9. Wash the cuvette with 10 mM HCl for 3 min to remove the non-covalently bound ligand to the matrix.
10. Wash with PBST for 5 min to get the baseline and to determine the amount of protein A.
11. Incubate the first concentration of HIgG in PBS/T for 30 min.
12. Wash with PBS/T to get the baseline.
13. Regenerate the surface with 10 mM HCl for 3 min.
14. Re-establish baseline with PBS/T
15. Repeat **steps 11–14** with different concentrations of HIgG (**Fig. 7a**) (*see* **Note 31**).
16. Plot dR/dt vs. R if the maximum binding response is known and the association between the two interactants is carried out under pseudo first order conditions for which the overall rate is:

$$\frac{dR}{dt} = k_{ass}(R_{max} - R)[C] - k_{diss}R,$$

$$\frac{dR}{dt} = k_{ass}(R_{max}[C] - R(K_{ass}[C] + K_{diss}).$$

However, if the maximum response is not known the k_a can be obtained by plotting the slope, k_s, of the plot dR/dt vs. R (**Fig. 7b**) for all concentrations of HIgG. The slope of this new plot is the k_a and the intercept, in theory, is the dissociation rate constant (k_d) (**Fig. 7c**).

$$K_s = k_a C + k_d \quad .$$

K_a was calculated for HIgG and protein A $1.13 \times 10^5 M^{-1}s^{-1}$ and the intercept value, k_d, was $1.83 \times 10^{-4}s^{-1}$.

Another way to determine the dissociate rate constant, which is independent of concentration, is to replace the sample HIgG with buffer and follow the dissociation phase. The dissociation

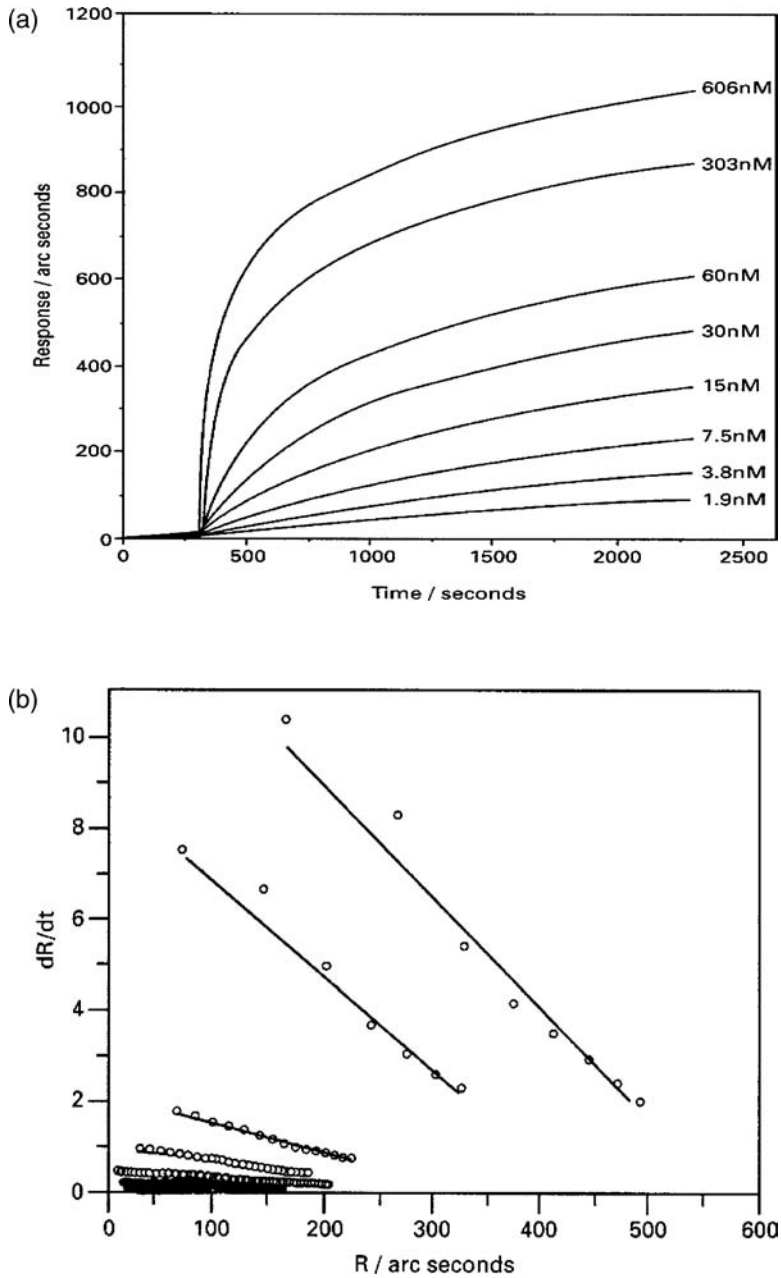


Fig. 7. (**a**) The biding curve of HIgG to immobilized SpA. (Reproduced from **ref.** *59* with permission from NeoSensors Ltd.). (**b**) Plots of dR/dt *vs* R for all HIgG concentrations. (Reproduced from **ref.** *59* with permission from NeoSensors Ltd.). (**c**) Slope K_s of d*R*/d*t* vs. *R* plotted against HIgG concentration. Determination of k_a for SpA:HIgG. (Reproduced from **ref.** *52* with permission from NeoSensors Ltd.). (**d**) Plot of $\ln(R_0/R_n)$ vs. $t_n - t_0$ to determine the dissociation rate constant for protein A:IgG. (Reproduced from **ref.** *59* with permission from NeoSensors Ltd.).

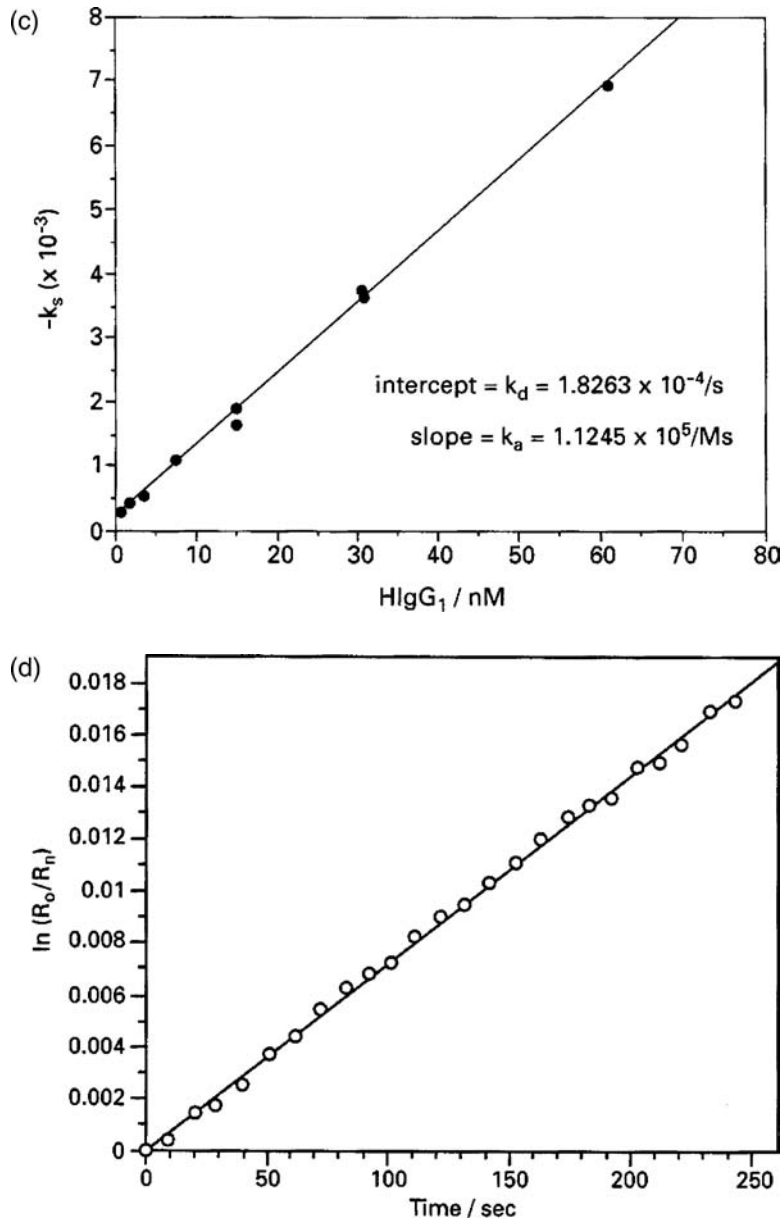


Fig. 7. (continued)

phase should follow the following equation, when rebinding is negligible due to complete removal of the dissociating analyte:

$$\frac{dR}{dt} = -K_{diss}R.$$

Integrating the above equation gives:

$$\frac{dR}{dt} = k_d(t_n - t_0),$$

where R_0 is the response at t_0, and R_n is the response at t_n. By constructing a plot of $\ln(R_0/R_n)$ vs. $t_n - t_0$, k_d is determined from the slope of the plot **(Fig. 7d)**. The k_d value was found to be 1.09×10^{-4}.

The overall affinity constant (k_d/k_a) of the HIgG and protein A was therefore calculated to be 1.62×10^{-9} M.

4. Notes

1. Silanization can be carried out by exposing the cleaned RM chip to 3-mainopropyletriethoxysilane vapour at 145 °C and about 3 mm Hg for 1 h *(2)*.
2. NHS coupling kit is commercially available from Neosensors (Durham, UK, CUV999900) if you do not want to prepare it. EDC/NHS aliquots should be stored at or below −18 °C.
3. The use of coherent illumination proved troublesome, as dust particles within the instruments caused light scattering, producing a characteristic speckle pattern on the detector.
4. The finite bandwidth of these sources has the effect of broadening the apparent width of the resonance peaks in the angular scan mode, while the finite size of the sources reduces the degree of collimation, which also broadens the peak in the wavelength scan mode.
5. For the angular scan, an interference filter with 670 ± 10 nm wavelength, 25 mm in diameter (Comar instruments, Cambridge, UK), and in case of wavelength scan a band-pass interference filters of wavelengths 512, 531, 550, 570, 596, 605, 632, 645 and 670 nm (10 nm FWHM bandwidth, Ealing Electro-optics, Watford, UK) was used to provide light of different wavelengths.
6. Fresh dissolved EDC should be used. In case degraded EDC has been used the cuvette can be treated with 1 M Tris, 3 M NaCl, pH 8.0 to release any accumulated materials.
7. In case of highly charged ligand or ligates, it is desirable to reduce the charges on the matrix by activating the matrix with EDC and NHS, followed directly by blocking with ethanolamine prior to carrying out the protocol as written.
8. The pH of the acetate buffer should be below the PI of the protein ligand, but above a value of 3.5.
9. 1 M Tris–HCl, pH 8.0 may also be used to block the unreacted NHS ester.

10. Avoid exposing the AS surface to buffers containing detergent prior to immobilization as it may reduce or variable the levels of ligand immobilization.
11. High concentration of ligands are needed as there is very little electrostatic concentration of ligand to the As surface unlike CMD surface.
12. Smaller and less charged proteins such as β-casein or cytochrome *c* can be used. Be sure that the sensor surface is completely coated as failure to coat the surface with protein results in an increase in the non-specific binding when ligates are added.
13. It is advisable after the PBST wash to further treat the immobilized avidin layer with the same regenerants that may be used in subsequent ligate binding experiments.
14. Streptavidin and NeutrAvidin from Pierce can be used instead of avidin.
15. Biotin cuvette can be reused several times after removing the avidin layer by treating the surface for 2 min with saturated KOH outside the instrument. It is essential to wash the surface thoroughly and rapidly to remove all traces of KOH prior to reuse.
16. Prevent de-wetting of the sensor surface by extensive washing.
17. To obtain reproducible results it is essential that the lipids used are not oxidized. It is recommended that the stock lipid solution contain 0.1% (w/w) butylated hydroxytoluene and are stored at less than −60 °C for no longer than 2 weeks.
18. It is recommended in case of using saturated lipids to wash the surface with 50% sucrose (w/w) in water before washing back into the PBS/AE. The sucrose solution will float and washed away the formed sticky micelles instead of the formation of a monolayer.
19. The resonance scan should be a symmetrical peak, indicating uniformity immobilization throughout the sensed area. If the peak is not symmetrical, stages 6–9 can be repeated.
20. The PLs can be stripped off from the surface by incubating the cuvette with 2% OG in high quality water for 3 min followed by washing with PBS.
21. Incubation time can be extended up to 15 min to ensure that a reasonable quantity of NTA is immobilized.
22. Due to the expensive nature of the TNA, it is recommended to recover NTA solution as it can be re-used.

23. It is recommended to extend the blocking time due to the extension of the activation time.
24. Alternatively, the sample can be removed by washing with 10 mM HEPES, 0.15 M NaCl, 0.35 M EDTA, 0.005% Tween 20, pH 8.3.
25. This procedure can be used at any time during the experiment to test the activity of the surface. The protocol was also found to work with FLAG-Ubiquitin (Sigma, U5382).
26. No significant non-specific binding of a digoxin-protein conjugate was observed.
27. The dissociation equilibrium constant for the interaction of peptide bFGF (127–140) with heparin is a 1,000-fold higher than that observed for native bFGF, its association rate constant and dissociation equilibrium constants for the peptide were readily determined by the RM. The results showed how RM can be used to analyze protein and a very low affinity peptide–carbohydrate interactions and provides evidence that a part of the recognition site of bFGF for heparin lies within the amino-acid sequence of 127–140 of bFGF *(43)*.
28. The results from **Fig. 3** show the specific hybridization of complementary oligonucleotides (Oligo-2 to immobilized Oligo-1) under average stringency conditions, while the non-complementary DNA (Oligo-3) did not give any significant response.
29. The RM is unsuitable for cell detection due to the short penetration of the evanescent field at the sensor surface, which places the majority of the cells outside the field *(59)*. However, the sensitivity of the RM technique can be increased 1,000-fold using a human immunoglobulin G (HIG)-colloidal gold complex *(19)*.
30. Keep cells in ice between the bindings.
31. All experimental data shown were generated on a single cuvette.

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