



Review

Surface plasmon resonance spectroscopy for characterisation of membrane protein–ligand interactions and its potential for drug discovery[☆]

Simon G. Patching^{*}

School of Biomedical Sciences and Astbury Centre for Structural Molecular Biology, University of Leeds, UK

ARTICLE INFO

Article history:

Received 27 February 2013
 Received in revised form 25 April 2013
 Accepted 29 April 2013
 Available online 9 May 2013

Keywords:

Surface plasmon resonance
 Membrane protein
 Ligand binding
 Sensors
 Kinetics
 Drug discovery

ABSTRACT

Surface plasmon resonance (SPR) spectroscopy is a rapidly developing technique for the study of ligand binding interactions with membrane proteins, which are the major molecular targets for validated drugs and for current and foreseeable drug discovery. SPR is label-free and capable of measuring real-time quantitative binding affinities and kinetics for membrane proteins interacting with ligand molecules using relatively small quantities of materials and has potential to be medium-throughput. The conventional SPR technique requires one binding component to be immobilised on a sensor chip whilst the other binding component in solution is flowed over the sensor surface; a binding interaction is detected using an optical method that measures small changes in refractive index at the sensor surface. This review first describes the basic SPR experiment and the challenges that have to be considered for performing SPR experiments that measure membrane protein–ligand binding interactions, most importantly having the membrane protein in a lipid or detergent environment that retains its native structure and activity. It then describes a wide-range of membrane protein systems for which ligand binding interactions have been characterised using SPR, including the major drug targets G protein-coupled receptors, and how challenges have been overcome for achieving this. Finally it describes some recent advances in SPR-based technology and future potential of the technique to screen ligand binding in the discovery of drugs. This article is part of a Special Issue entitled: Structural and biophysical characterisation of membrane protein–ligand binding.

© 2013 Elsevier B.V. All rights reserved.

Contents

1. Introduction	44
2. The surface plasmon resonance experiment	44
3. Challenges for characterising membrane protein–ligand interactions using SPR	46
4. Applications with membrane protein systems	46
4.1. GPCRs	46
4.1.1. Rhodopsin	46
4.1.2. Chemokine receptors CCR5 and CXCR4	47
4.1.3. Neurotensin receptor-1	49
4.1.4. Human olfactory receptor 17-4	49

Abbreviations: ABC, ATP binding cassette; ADP, Adenosine-5'-diphosphate; AMP, Adenosine-5'-monophosphate; AMPPNP, Adenosine-5'-(β,γ -imido)triphosphate; ATP, Adenosine-5'-triphosphate; BACE1, β -Site amyloid precursor protein cleaving enzyme 1; BPM, Biophysical Mapping; CHAPSO, 3-[(3-Cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate; CMC, Critical micelle concentration; DDM, *n*-Dodecyl- β -D-maltoside; EOT, Extraordinary optical transmission; EGF, Epidermal growth factor; GABA, γ -Aminobutyric acid type A (receptors); GDP, Guanosine-5'-diphosphate; GPCR, G protein-coupled receptor; GTP, Guanosine-5'-triphosphate; hOR17-4, Human olfactory receptor 17-4; HPA, Hydrophobic association (sensor chip); hPRR, Human (pro)renin receptor; HTA, ω -Hydroxy-undecanethiol; MSP, Membrane scaffold protein; N-Y4, Neuropeptide Y4; NPY, Neuropeptide Y; PDB, Protein Data Bank; POPC, 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; PP, Pancreatic polypeptide; PYY, Polypeptide YY; RU, Resonance or response units; SAM, Self-assembled monolayer; SDF-1 α , Stromal cell-derived factor 1 α ; SLB, Supported lipid bilayer; SPR, Surface plasmon resonance; SPRM, Surface plasmon resonance microscopy; StaR, Stabilised receptor

[☆] This article is part of a Special Issue entitled: Structural and biophysical characterisation of membrane protein–ligand binding.

^{*} Astbury Building, Faculty of Biological Sciences, University of Leeds, Leeds, LS2 9BS, UK. Tel.: +44 1133433129.

E-mail address: s.g.patching@leeds.ac.uk.

4.1.5.	Neuropeptide Y4 receptor N-terminal domain	49
4.1.6.	Adenosine-A _{2A} receptor	49
4.1.7.	β ₁ -Adrenergic receptor	49
4.2.	Non-GPCRs	50
4.2.1.	Outer membrane receptor FhuA	50
4.2.2.	Tyrosine kinase HER2 receptor subdomain	51
4.2.3.	Human (pro)renin receptor	51
4.2.4.	α-Hemolysin	51
4.2.5.	β-Site amyloid precursor protein cleaving enzyme 1	51
4.2.6.	Human CD4 receptor in nanodiscs	51
4.2.7.	Human ABC transporter P-gp in nanodiscs	52
4.2.8.	Epidermal growth factor receptor on intact cells	52
4.2.9.	β3 γ-aminobutyric acid type A receptors	52
5.	Recent developments and potential for drug discovery	53
6.	Conclusions	54
	Acknowledgements	54
	References	54

1. Introduction

Membrane proteins are coded by up to 30% of the open reading frames in known genomes [1–3], they have important roles in many biological processes (e.g. transport of ions and molecules, control of transmembrane potential, generation and transduction of energy, signal recognition and transduction, catalysis of chemical reactions) and mutations in membrane proteins have been linked with a number of human diseases [4–10]. The molecular targets for around 50–60% of current validated medicines are membrane proteins and they remain the principal target for new drug discovery [11–17]. Owing to the difficulties in applying the main biophysical techniques for high-resolution protein structure determination: X-ray crystallography and NMR spectroscopy, the number of structures of membrane proteins is still relatively few, contributing less than 1% of protein structures in the Protein Data Bank (PDB) [18], thus limiting the amount of information available for traditional structure-based drug design. At the time of writing, there are high-resolution structures determined for only seventeen unique G-protein-coupled receptors (GPCRs) [19], which represent the largest class of membrane protein drug target. Other membrane protein drug targets include cytokine receptors, tyrosine and histidine kinase receptors, antibody receptors, ligand- and voltage-gated ion channels and transport proteins. It is important to have a range of chemical, biochemical and biophysical techniques available for characterisation of ligand binding by membrane proteins and for screening libraries of compounds as potential drug candidates. A developing technique in this respect is surface plasmon resonance (SPR) spectroscopy, which is label-free and enables measurement of real-time quantification of ligand-binding affinities and kinetics using relatively small amounts of membrane protein in a native or native-like environment and has potential to be medium-throughput. Following a description of the SPR experiment, this review first considers the challenges associated with applying SPR-based methods to characterise ligand binding by membrane proteins and then demonstrates how some of these have been overcome with examples of its application to a range of specific membrane protein systems. In some cases, this involves combination with results from other experimental techniques and with molecular modelling. Finally it describes some recent developments in SPR-based technology and considers its future potential for drug discovery with membrane protein targets.

2. The surface plasmon resonance experiment

Surface plasmon resonance (SPR) uses an optical method to measure a change in refractive index of the medium in close vicinity of a metal surface that can be used to monitor the binding of analyte molecules to receptor molecules immobilised on the metal surface

[20,21]. This exploits the phenomenon of surface plasmon generation in thin metal films and the total internal reflection of light at a surface-solution interface to produce an electromagnetic film or evanescent wave that extends a short distance (up to 300 nm) into the solution (see other reviews for a more detailed description of the theory behind surface plasmon generation [22–27] and references therein). SPR has predominantly been developed and performed using BIACore™ technology [20,28–36] with the first commercial instrument in 1991; an illustration of the basic instrument set up is shown in Fig. 1A. The surface is typically a thin film of gold on a glass support that forms the floor of a small-volume (less than 100 nl) flow cell through which an aqueous solution is passed continuously. In order to detect the binding of an analyte molecule to a receptor molecule, the receptor molecule is usually immobilised on the sensor surface and the analyte molecule is injected in the aqueous solution through the flow cell. Polarised light from a laser source is directed through a prism to the under surface of the gold film where surface plasmons are generated at a critical angle of the incident light. This absorption of light is seen as a decrease in intensity of the reflected light. The critical angle is dependent on the refractive index of the medium within 300 nm of the gold surface and changes when molecules bind to the surface, e.g. when analyte molecules bind to immobilised receptor molecules (Fig. 1B). The real-time response of the SPR experiment is usually presented in the form of a sensorgram (Fig. 1C). If interaction between the immobilised receptor molecules and the analyte molecules occurs, the refractive index at the surface of the gold film changes and this is seen as an increase in signal intensity. Resonance or response units (RU) are used to describe the increase in the signal, where 1 RU is equal to a critical angle shift of 10^{-4} deg. At the start of the experiment all immobilised receptor molecules have not been exposed to analyte molecules and the RU value corresponds to the starting critical angle a . Analyte molecules are injected into the flow cell; if they bind to the immobilised receptor molecules, there is an association phase during which binding sites become occupied and the shape of this curve can be used to measure the rate of association (k_{on}). When steady-state is achieved the RU value corresponds to the changed final critical angle b . This maximum RU value relates to the concentrations of immobilised receptor and analyte molecules and so can be used to measure the binding affinity (K_D). When analyte molecules are removed from the continuous flow there is a dissociation phase during which binding sites become unoccupied and the shape of this curve can be used to measure the rate of dissociation (k_{off}). The surface can then be regenerated and returned to the critical angle a to start the experiment again. The lowest detectable concentration in the SPR experiment depends on a number of factors including the molecular weight, optical property and binding affinity of the analyte molecule

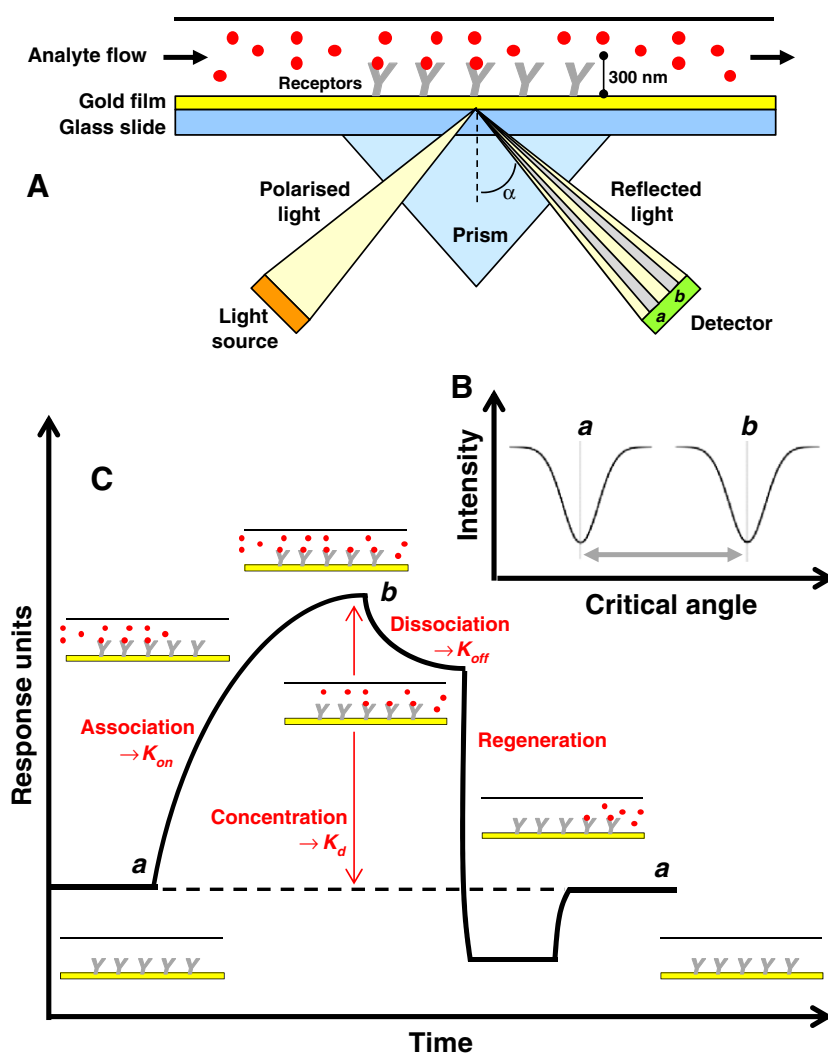


Fig. 1. Schematic illustration of the basic SPR experiment for measuring the binding of an analyte molecule to a receptor molecule. A. Instrument set up for an SPR experiment based on BIAcore™ technology. SPR uses an optical method to measure the refractive index near to a sensor surface; this exploits total internal reflection of light at a surface-solution interface to produce an electromagnetic field or evanescent wave that extends a short distance (up to 300 nm) into the solution. The surface is a thin film of gold on a glass support that forms the floor of a small-volume (less than 100 nl) flow cell through which an aqueous solution is continuously passed. In order to detect the binding of an analyte molecule to a receptor molecule, the receptor molecule is usually immobilised on the sensor surface and the analyte molecule is injected in the aqueous solution through the flow cell. Polarised light from a laser source is directed through a prism to the under surface of the gold film where surface plasmons are generated at a critical angle of the incident light. This absorption of light is seen as a decrease in intensity of the reflected light. The critical angle is dependent on the refractive index of the medium within 300 nm of the gold surface and changes when molecules bind to the surface, e.g. when analyte molecules bind to immobilised receptor molecules. B. Change in the critical angle of incident light from angle *a* to angle *b* on binding of an analyte molecule to a receptor molecule. C. Response of the SPR experiment in the form of a sensorgram. If interaction between the immobilised receptor molecule and the analyte molecule occurs, the refractive index at the surface of the gold film changes and this is seen as an increase in signal intensity. Resonance or response units (RU) are used to describe the increase in the signal, where 1 RU is equal to a critical angle shift of 10^{-4} deg. At the start of the experiment all immobilised receptor molecules have not been exposed to analyte molecules and the RU correspond to the starting critical angle *a*. Analyte molecules are injected into the flow cell; if they bind to the immobilised receptor molecules, there is an association phase during which binding sites become occupied and the shape of this curve can be used to measure the rate of association (k_{on}). When a steady-state is achieved (all binding sites occupied in this example) the RU correspond to the changed final critical angle *b*. This maximum RU relates to the concentrations of immobilised receptor and analyte molecules and so can be used to measure the binding affinity (K_D). When analyte molecules are removed from the continuous flow there is a dissociation phase during which binding sites become unoccupied and the shape of this curve can be used to measure the rate of dissociation (k_{off}). The surface can then be regenerated and returned to the critical angle *a* to start the experiment again. (This figure was constructed based on pictures and information given in references [20,21,27,40]).

as well as the surface coverage of the receptor molecule. The SPR response correlates with a change in mass concentration on the sensor chip surface and therefore depends on the molecular weight of the analyte molecule in relation to the number of receptor sites on the sensor surface. If the term R_{max} describes the maximum binding capacity of the surface receptor molecule for the analyte molecule in RU, the theoretical R_{max} is calculated using the equation $R_{max} = (MW_{analyte}/MW_{receptor}) \times R_{rec} \times V_{rec}$, where $MW_{analyte}$ is the molecular weight of the analyte molecule, $MW_{receptor}$ is the molecular weight of the receptor molecule, R_{rec} is the response obtained from the receptor molecule and V_{rec} is the valency of the receptor molecule/proposed stoichiometry of the interaction [37,38]. Achieving conditions

with an optimum R_{max} is important for measuring the binding kinetics of an interaction.

A range of 'sensor-chips' are commercially available for use with SPR instruments allowing the user to immobilise their receptor molecule of interest to the gold surface [20,39–42]. For example, the hydrophobic association (HPA) sensor chip contains long-chain alkanethiol molecules covalently attached to the gold surface. Vesicles are adsorbed on to the surface forming a supported lipid monolayer. Most chips other than HPA are based on carboxylated dextran surfaces to allow preconcentration and/or chemistry to be performed. For example, the L1 chip allows formation of lipid bilayers; its surface has a dextran matrix modified with hydrophobic anchors enabling

capture of vesicles that fuse and subsequently form a bilayer. Some have functional groups (e.g. amino, thiol, aldehyde or carboxyl) to enable use of specific chemistry for the covalent immobilisation of receptor molecules on to the surface. If it is not possible to directly immobilise the receptor molecule on to the surface, then a secondary molecule such as an antibody can be used. An antibody that recognises the receptor of interest is covalently immobilised on the surface using specific chemistry then the receptor molecule is 'captured' on to the surface by the antibody before exposure to analyte molecules. Since the sensor chips can usually be regenerated after each experiment, by washing off all analyte molecules, the same chip can be used to test for binding of a number of different analyte molecules.

3. Challenges for characterising membrane protein–ligand interactions using SPR

As with any structural or functional investigation with membrane proteins, use of SPR to characterise their interaction with ligands requires the protein to be in its original membranes or reconstituted in a suitable membrane mimetic or solubilised in a suitable detergent that retains the native structure, conformation and activity of the protein as far as possible. For characterisation of ligand binding, retaining activity is obviously of most importance. This challenge usually has to be combined with immobilisation or capture of the membrane protein on to the sensor surface. Some of the approaches that have been developed to achieve this, which include covalent attachment by selective chemistry and capture by antibodies or affinity tags combined with solubilisation and reconstitution strategies, are described along with application to specific membrane protein systems in the following section of this review.

Once the membrane protein is attached to the sensor surface, a suitable ligand molecule has to be chosen for introduction into the analyte flow to test for protein activity. The choice of ligand molecule will depend on the membrane protein system under investigation and the aim of the intended experiments, e.g. screening a specific binding site for ligand specificity. This should include appropriate control experiments to show that the observed ligand binding activity is specific, e.g. using a different membrane protein or a different ligand as a negative control. It may be useful to screen a range of lipid reconstitution or detergent solubilisation conditions for the membrane protein under investigation to identify those that give the highest protein activity and stability. The measured ligand binding activity and specificity determined from the SPR experiment can also be validated by applying other biochemical or biophysical techniques to the membrane protein reconstituted or solubilised under similar conditions, e.g. using a radioligand binding assay. The structural integrity of the protein can also be tested in a similar way. Such considerations are discussed along with application to specific membrane protein systems in the following section of this review.

Having the membrane protein attached to the sensor surface under lipid-reconstituted or detergent-solubilised conditions that retain the active conformation of the protein is an important consideration. A rigorous demonstration of ligand binding and activity, as described above, is a good indication of a membrane protein retaining its correct conformation. Further demonstration of a correct conformation can be achieved by observing the binding of conformation-dependent antibodies to the membrane protein of interest. A homogenous surface with all receptor molecules in the same orientation where the ligand binding site is directed towards the analyte flow rather than towards the sensor surface is also an important consideration and should assist efficiency of the experiment. This is achievable by capture methods that use affinity tags or antibodies where the receptor molecules are oriented by attachment from a common site, but this does need prior knowledge about the amino acid sequence and/or structure of the receptor molecule under investigation. For some membrane proteins, access of the ligand to both sides of the protein may be necessary to elicit the binding

response. This can be hindered if one side of the protein is used for attachment to the sensor surface, so experimental systems have been developed that do allow access to both sides. These considerations are described along with application to specific membrane protein systems in the following section of this review.

Since the SPR effect is due to detection of a mass change at the sensor surface, where binding of larger molecules will produce a greater change in refractive index, detecting the binding of small-molecule ligands is more challenging than for larger ones. Many membrane protein ligands of interest, especially in drug discovery, are small molecules with molecular weights of less than 1000 Da. The detection of small-molecule ligands by SPR to membrane proteins is made easier by having a high protein density on the sensor surface, but care has to be taken that the protein is still active since a high density of denatured protein is not very useful. When using the SPR experiment to screen a number of ligands binding to a membrane protein attached to a sensor surface, either directly or by competition with another ligand, it is important to have appropriate washing steps that regenerate the sensor surface to its original condition and protein activity before introduction of the next ligand. It is also important that the activity and stability of the protein is retained for the duration of an experiment that screens for the binding of a number of ligands using the same sensor surface. This can be kept in check by performing appropriate activity and control measurements throughout the experiment, including at the end. Conditions where there is minimal time-dependent loss of protein activity are obviously desirable. These are further challenges that have to be considered, some of which have been overcome as described along with application to specific membrane protein systems in the following section of this review.

4. Applications with membrane protein systems

4.1. GPCRs

The abundance and importance of GPCRs and their roles as drug targets has been described in other contributions to this Special Issue. SPR methods have been developed and used to characterise ligand binding with a number of GPCR systems, which are described in this section.

4.1.1. Rhodopsin

Some of the earliest works that used SPR to detect and characterise binding to a GPCR, indeed to any membrane protein, were performed on the light-activated receptor rhodopsin. Salamon et al. incorporated bovine rhodopsin into an egg phosphatidylcholine bilayer deposited on a thin metal film, in this case silver, and demonstrated the tight binding and activation of its associated G-protein (transducin) from the SPR data [43]. It was possible to monitor and quantify the saturable binding of transducin to the receptor and then follow effects from a light-induced conformational change and then binding of GTP on its addition to the aqueous phase.

A few years later, spatially and time-resolved SPR measurements and then a micropatterned immobilisation technique were developed [44,45] to enable G protein activation, ligand binding, and receptor deactivation with bovine rhodopsin to be followed by SPR (Fig. 2). The key to the latter approach was use of microcontact printing to produce micrometer-sized patterns that had high contrast in receptor activity compared with the background and therefore enhanced sensitivity. Rhodopsin was immobilised on the sensor surface by exploiting a glycosylation site at the extracellular N-terminus that is conserved among GPCRs and use of carbohydrate-specific chemistry for biotinylation (Fig. 2). Streptavidin was bound to biotinylated-thiols in a mixed self-assembled monolayer (SAM) with an excess of ω -hydroxy-undecanethiol (HTA) on the metal surface, which then bound the biotinylated receptor to the surface through its extracellular N-terminus in a defined orientation (Fig. 2). Following immobilisation of the receptor and thorough washing with

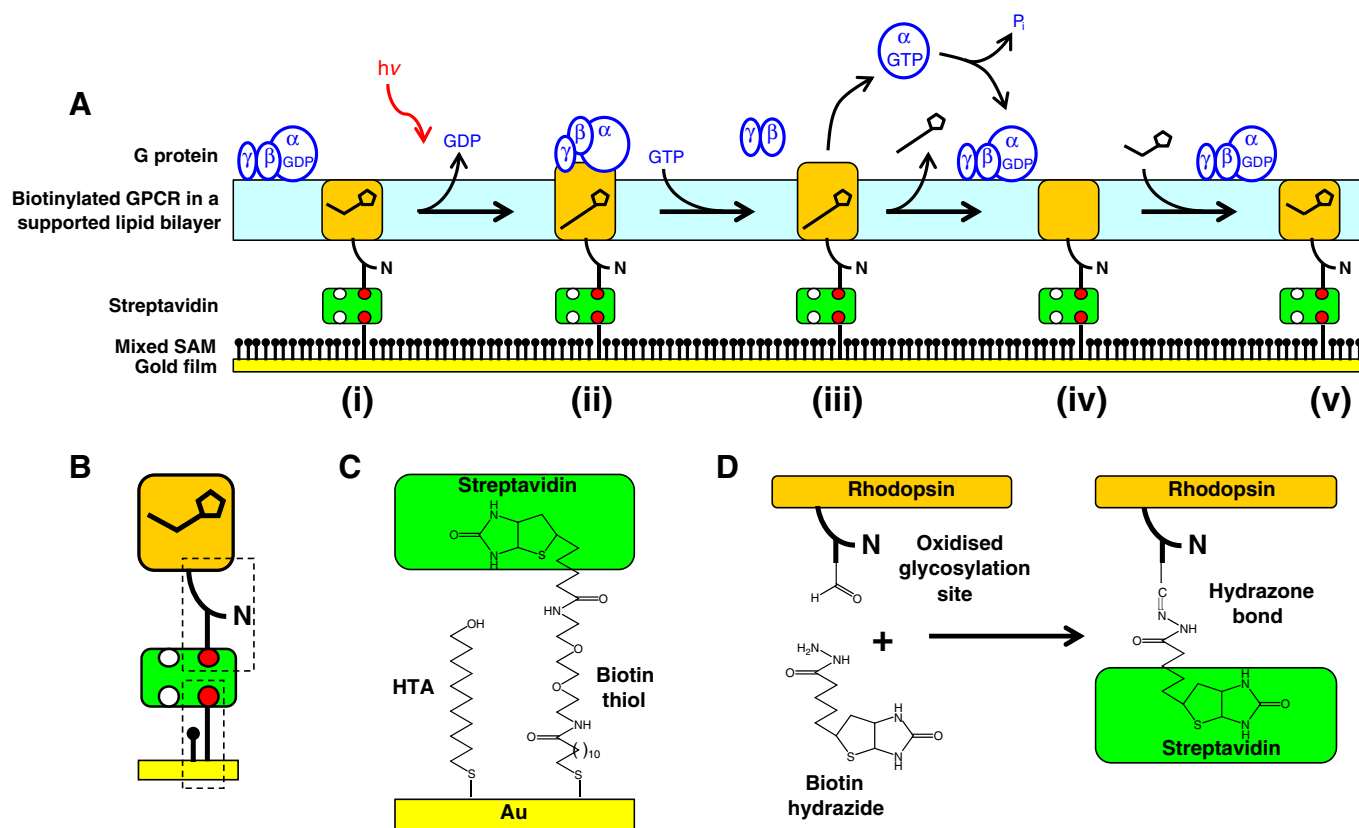


Fig. 2. Immobilisation of rhodopsin for monitoring G protein activation, ligand binding and receptor deactivation events by SPR. **A.** (i) On a gold film coated with a mixed SAM of biotinylated-thiols with an excess of ω -hydroxy-undecanethiol (HTA), streptavidin is bound which then binds the receptor through a carbohydrate-specific biotinylation site at its N-terminus. A supported lipid bilayer is then formed around the immobilised receptor, which binds the G-protein. (ii) Light-induced photoisomerisation of receptor-bound 11-*cis* to all-*trans*-retinal triggers the active conformation of rhodopsin, which binds the G-protein releasing its GDP. (iii) The G-protein desorbs from the receptor upon GTP binding. (iv) The activated receptor decays spontaneously to all-*trans*-retinal and opsin and the G-protein binds again to the membrane surface following hydrolysis of GTP. (v) *cis*-retinal binds to opsin, which regenerates photoactivatable rhodopsin. **B.** Expansion of the arrangement on the sensor surface with hatched boxes highlighting the regions expanded further in C and D along with details of the surface chemistry. **C.** Mixed SAM of HTA and the biotinylated thiol 12-mercaptododecanoic-(8-biotinoylamido-3,6-dioxaoctyl)amide, which are covalently attached to the gold surface through their sulphur atom; the latter binds streptavidin through its biotin group. **D.** A glycosylation site on the extracellular N-terminus of rhodopsin is oxidised and then reacted with biotin hydrazide to form a hydrazone bond; the biotinylated receptor is then bound to streptavidin that is already attached to the preformed SAM on the sensor surface. (This figure was constructed based on a picture and information given in Bieri et al. [45]).

detergent (48 mM), a supported lipid bilayer (SLB) [46] was formed around the receptors by use of a detergent micellar dilution method [47]. This involved treatment with an aqueous solution of 0.1 M KCl containing 50 mM the detergent octyl glucoside and 4 mM of phosphatidylcholine lipid. Formation of the lipid layer was achieved by stepwise dilution of the detergent in the analyte flow to below its CMC value and until the SPR response was stable. Having this lipid layer supported on top of the preformed SAM provides a water layer between the two; this is important for accommodating the proper folding of extramembrane parts of the reconstituted receptor. Micropatterns of SAMs on the metal surface with alternating stripes (width 200 μm) of pure HTA (background reference) and biotin-thiol/HTA (receptor binding region) were produced followed by the binding procedure described above. Activity of the immobilised receptor was observed in SPR data following its illumination with light, which was achieved by use of home-built SPR equipment where a glass window at the opposite side of the cuvette allowed for flash illumination of the surface (see reference [44] for a diagram of the optical configuration). Illumination induced activity of the G protein was followed by its desorption from the membrane (Fig. 2A, (ii) and (iii)); this activity was twenty five times lower in the reference region of the micropattern. Following activation, the SPR data could also then follow cleavage of the Schiff's base between rhodopsin and its chromophore all-*trans*-retinal and relaxation of the G protein to its starting position (Fig. 2A, (iv) and (v)). Ligand binding was monitored and quantified from the SPR data by adding 11-*cis*-retinal in increasing concentrations to

the immobilised and completely photolysed receptor (opsin), which gave a dissociation constant of 130 nM.

In further experiments using rhodopsin as a model protein, Karlsson and Löfås developed a rapid flow-mediated on-surface immobilisation and reconstitution method for SPR measurements with membrane proteins [48]. This used a carboxylated dextran surface modified with long alkyl groups (L1 chip) to which detergent-solubilised purified receptor was immobilised by amine-coupling. The surface was immediately washed with lipid/detergent (POPC/octylglucoside) mixed micelles then the detergent was eluted in the subsequent buffer flow and the remaining lipid formed a bilayer on the sensor surface, which reconstituted the receptor. Activity of the reconstituted receptor was demonstrated by monitoring the rhodopsin-mediated dissociation of transducin. Since the reconstitution procedure could be achieved in approximately 1 minute and the deposited lipids could be completely removed by two consecutive injections of detergent, this method offered potential for medium-throughput measurements with membrane proteins that are stable to the procedure.

4.1.2. Chemokine receptors CCR5 and CXCR4

SPR has been used to characterise ligand binding to the human chemokine receptors CCR5 and CXCR4. These receptors have also been used to demonstrate important developments in SPR methods for purification, solubilisation, reconstitution and functional analysis of GPCRs. Hüttenrauch et al. used SPR to investigate the location of

β -arrestin 1 binding to CCR5, which was shown to be at a conserved Asp-Arg-Tyr motif within the second intracellular loop [49]. This work used C-terminal derived peptides and a cytoplasmic loop of CCR5 immobilised on a CM5 sensor chip through the thiol group of an N-terminal cysteine residue or on a Sa5 streptavidin sensor chip through an N-terminal biotin moiety, respectively. β -Arrestin 1 was included in the analyte flow for analysis of its binding from the SPR response.

Using CCR5 and CXCR4 as model systems, Stenlund et al. [50] developed a method for the capture and reconstitution of GPCRs on to a sensor surface from crude cell preparations without the need for their prior purification (Fig. 3A). The 'capture and reconstitution' method first involves immobilisation of a capturing molecule that recognises the GPCR at a site distinct from the ligand binding site, in this case 1D4 monoclonal antibodies were immobilised through aldehyde coupling chemistry to a hydrazide-modified L1 sensor chip. Detergent-solubilised receptors

from crude cell lysates are then captured by the antibody molecules on to the sensor surface. Washing with lipid/detergent mixed micelles, in this case made of POPC/CHAPSO, reconstitutes the receptors in a lipid bilayer from which detergent is removed by washing with buffer (Fig. 3B) [50]. The captured and reconstituted receptors can then be tested for activity and for the binding of ligands. Both CCR5 and CXCR4 were captured in this way then the structural and functional integrity of CXCR4 was tested by the binding of a conformationally-dependent antibody and a native chemokine ligand stromal cell-derived factor 1 α (SDF-1 α) (Fig. 3C and D) [50]. Having the receptor molecule attached to the antibody at a known site that is distinct from the ligand binding site helps to orient the receptor so that the ligand binding site is facing towards the analyte flow rather than towards the sensor surface and should also create a more homogenous surface. Interestingly, binding of SDF-1 α to CXCR4 captured and reconstituted on an L1 chip, captured on an L1 chip without lipids and captured on a CM5 chip gave similar

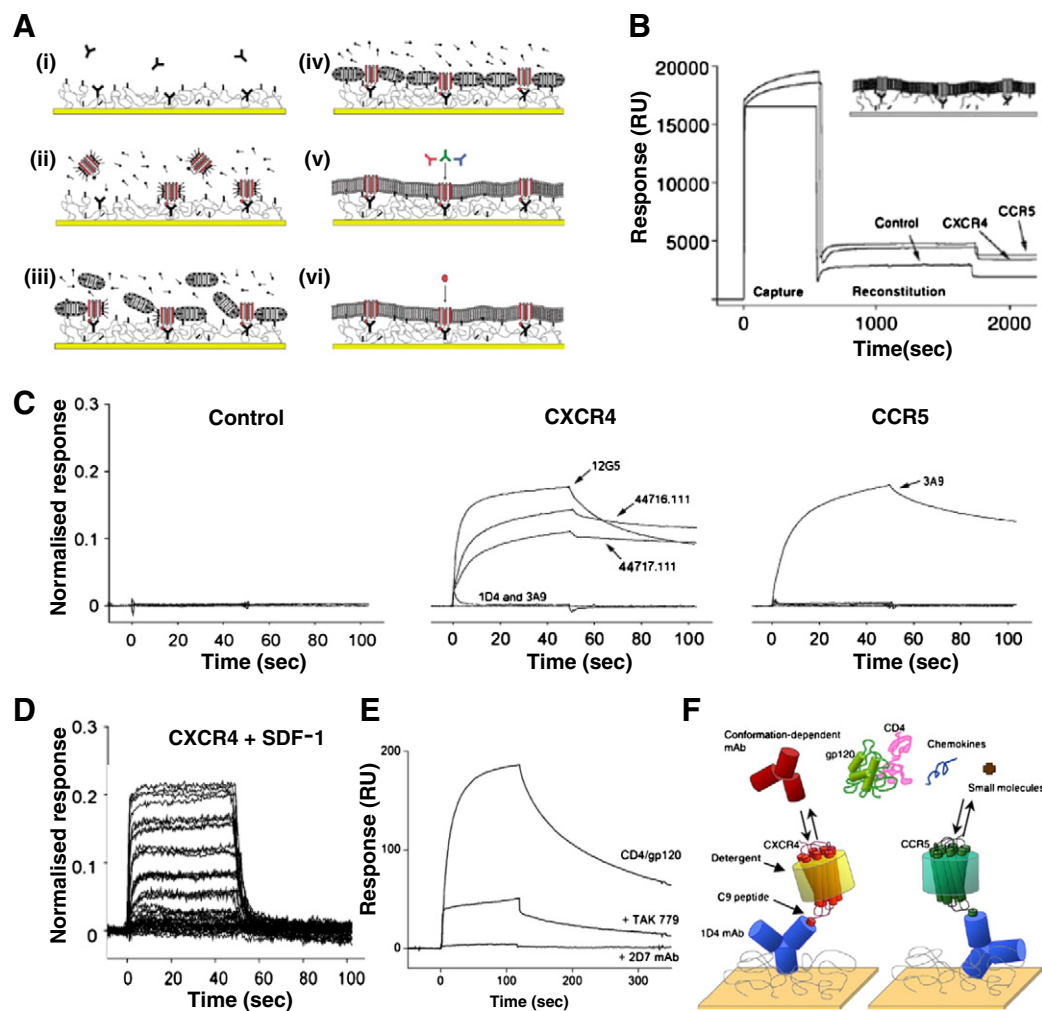


Fig. 3. Capture and reconstitution of GPCRs on a biosensor surface: Binding of conformation-dependent antibodies and small-molecule ligands to the chemokine receptors CXCR4 and CCR5. **A.** Schematic illustration for the capture and reconstitution of GPCRs on a sensor surface. (i) A capturing molecule that recognises the GPCR at a position distant from the ligand binding site, in this case 1D4 monoclonal antibody, is immobilised on an L1 sensor chip that has a dextran surface containing hydrophobic alkane groups. (ii) A detergent-solubilised GPCR is captured by the immobilised antibody. (iii) The captured GPCR is reconstituted in a lipid bilayer by injecting lipid/detergent-mixed micelles in the analyte flow. (iv) The surface is washed with buffer to remove detergent molecules, leaving behind a lipid bilayer. (v) The functional activity of the lipid-reconstituted GPCR is tested by binding of conformation-dependent antibodies. (vi) Binding of small-molecule ligands by the captured and reconstituted GPCR can then be tested. **B.** Sensorgrams illustrating the capture and reconstitution of CXCR4 and CCR5 receptors in POPC lipids on an L1 sensor chip from detergent-solubilised Cf2Th cells. **C.** Sensorgrams illustrating the binding of 1D4 antibody, conformation-dependent monoclonal antibodies (12G5, 44716.111, 44717.111) and anti-CCR5 antibody 3A9 to control, CXCR4 and CCR5 sensor surfaces with the receptors captured and reconstituted in POPC lipids on an L1 chip. **D.** Sensorgrams illustrating the binding of chemokine SDF-1 α at a range of concentrations (0, 1.25, 2.5, 5, 10, 20, 40, 80, 160, 320, 640 nM) to CXCR4 captured and reconstituted in POPC lipids on an L1 sensor chip. **E.** Sensorgrams illustrating the inhibition of gp120/CD4 binding to CCR5 by the small-molecule TAK-779 (10 μ M) and the 2D7 monoclonal antibody (156 nM), where responses are compared with uninhibited binding of gp120/CD4 (100 nM). **F.** Schematic illustration of the capture and reconstitution method for measuring the binding of conformation-dependent antibodies and small-molecule ligands to chemokine receptors CXCR4 and CCR5. (Pictures A–D are modified from Stenlund et al. [50], E is modified from Navratilova et al. [51] and F is reproduced from Navratilova et al. [52]).

dissociation equilibrium constants (K_D) of 160 ± 3 , 156 ± 2 and 180 ± 4 nM, respectively, which were also similar to a value of ~ 200 nM determined from a cell-based assay. This demonstrates that a membrane protein may not have to be in a membrane or lipid environment to retain its ligand binding activity, solubilisation in detergent may be sufficient; this has to be tested on a case by case basis with each membrane protein under investigation. The same group made a number of further developments to the method and successfully demonstrated its use for screening small-molecule binding. An automated BIAcore™-based assay was developed for screening receptor solubilisation conditions to improve receptor activity and stability, which was demonstrated with CCR5 and CXCR4 [51]. This work also demonstrated that CCR5 was functional with respect to binding the HIV-1 viral surface protein gp120, which was inhibited by the small molecule TAK-779 (Fig. 3E) [51]. The system was then used to screen for further small molecule binding (Fig. 3F) [52], as follows. CCR5 and CXCR4 were captured by 1D4 immobilised on a CM4 sensor chip using amine-coupling chemistry and their activities were demonstrated by binding of the native chemokine ligands RANTES and SDF-1 α , respectively. Nineteen small-molecule inhibitors (average MW 550 Da) at a range of concentrations were tested for binding to CCR5 using freshly prepared sensor surfaces. The resultant binding affinities (K_D values) from the SPR measurements showed good correlation with inhibition constants (K_i values) obtained from a whole-cell based assay that tested binding of the same compounds [52]. This work therefore demonstrated the potential for using SPR to screen small-molecule libraries of compounds for their binding to GPCRs.

In the same year, Silin et al. reported an alternative method for capturing GPCRs on a sensor surface using CCR5 as a model [53]. This involved selective immobilisation of receptor-containing membrane vesicles on a sensor surface that was constructed from sequential treatments of biotin in a protein-resistant matrix with (strept)avidin, a biotinylated antibody, and a receptor-specific antibody.

The automated BIAcore™ technology was also used to develop an affinity purification method and a screen for co-crystallisation conditions with CCR5 [54]. This work included characterisation of nine HIV-1 gp120 variants and identified a truncated construct that bound CCR5 independent of CD4, which was then used in an affinity purification step to improve activity of the detergent-solubilised receptor by approximately 300% [54]. Automated systems for detergent screening of GPCRs were also developed using CCR5 [55]. The developed SPR methods with CCR5 have been used to measure the real-time binding of gp120 and to identify antagonists that bind to the receptor and stabilise a conformation that is unable to bind the HIV-1 gp120–CD4 complex [56] and to screen for the binding of novel orthosteric and allosteric ligands [57]. In recent work that demonstrated CCR5 to be a receptor for *Staphylococcus aureus* leukotoxin ED [58], SPR was used to show a direct interaction between the LukE subunit and CCR5 in which binding was time-dependent and saturable with an apparent dissociation constant (K_D) of 39.6 ± 0.4 nM. An inability of LukE to bind to CXCR4 confirmed the binding to CCR5 to be specific.

4.1.3. Neurotensin receptor-1

A novel receptor-analyte configuration was used to characterise neurotensin receptor-1 binding to the neurotransmitter peptide neurotensin using SPR [59,60]. Neurotensin biotinylated at the N-terminus was immobilised on a streptavidin-coated sensor chip and purified detergent-solubilised receptor was included in the analyte flow for analysis of the receptor-ligand interaction. The reasoning behind this arrangement was that binding of the larger receptor molecule to the immobilised ligand would produce a greater mass change on binding and therefore a larger SPR response. An N-terminal biotinylated 'scrambled' peptide with the same residues as neurotensin was used to create a control sensor surface for receptor binding. A specific concentration-dependent ligand-receptor interaction was demonstrated from the SPR data, which yielded an apparent K_D value of 1–2 nM similar to values measured using a radioligand-binding assay.

4.1.4. Human olfactory receptor 17-4

SPR has been used to demonstrate the ligand binding activity of a human olfactory receptor produced by cell-free synthesis [61]. Human olfactory receptor 17-4 (hOR17-4) was captured by a monoclonal anti-polyhistidine antibody immobilised on a CM4 sensor chip using amine-coupling chemistry. The odorant undecanal was injected at a range of concentrations and the resultant SPR response was used to derive a binding affinity of ~ 22 μ M, which was in agreement with measurements obtained from other *in vitro* techniques.

4.1.5. Neuropeptide Y4 receptor N-terminal domain

As part of structural and functional studies of the 41-residue N-terminus of the neuropeptide Y4 receptor (N-Y4), SPR was used to investigate possible interactions with peptides from the neuropeptide Y (NPY) family [62]. N-terminal biotinylated neuropeptides were immobilised on a streptavidin-coated sensor chip and N-Y4 was injected in the analyte flow at a range of concentrations. The SPR response gave a K_D value of 50 μ M for binding of the natural ligand pancreatic polypeptide (PP), whilst binding of the hormones neuropeptide Y (NPY) and peptide YY (PYY) was too weak to measure an affinity (> 1 mM).

4.1.6. Adenosine- A_{2A} receptor

Using the adenosine A_{2A} receptor, a new approach called 'Biophysical Mapping' (BPM) [63,64] has been developed that combines a thermostabilised GPCR with SPR analysis of ligand binding to binding-site mutants to give matrices of data that can be used to produce high quality three-dimensional pictures of ligand binding sites in the absence of a high-resolution crystal structure or can be combined with such a structure (Fig. 4). A stabilised form of the A_{2A} receptor, 'StaR' was engineered by introducing a number of mutations, which included A54L, T88A, K122A and V239A following alanine-scanning mutagenesis. Further single-site mutations were introduced into this StaR background at eight positions (L85A, L167A, M177A, N253A, Y271A, I66A, N181A, S277A) predicted to be directly involved in ligand binding from a homology model based on the crystal structure of the thermostabilised β_1 -adrenergic receptor and from the results of radioligand binding with the antagonist [3 H]ZM241385. Each of the detergent-solubilised StaR mutants was immobilised on a Ni-loaded NTA sensor chip through their His-tag and tested for binding with a library of 21 small-molecule compounds that were injected separately in the analyte flow at a range of concentrations (5–80 nM). The SPR responses (Fig. 4B) were used to create matrices of binding affinities and kinetic information (K_D , k_{on} , k_{off}) to compare the effects of each mutation on ligand binding specificity (Fig. 4C). Binding affinities measured for each compound with the unaltered StaR background were very similar to those obtained from a competitive radioligand-binding assay. The structure-activity relationships observed in the SPR data were used to create biophysical maps and to optimise homology models of the A_{2A} receptor binding site with docked ligands (Fig. 4D and E). A subsequent crystal structure of the A_{2A} receptor in complex with ZM241385 [65] allowed testing of the homology model with this ligand that was revised based on the BPM experiments. The binding pose of the ligand was very similar in the crystal structure and model except for a difference in the χ_1 angle of Tyr271. A later crystal structure solved for an A_{2A} StaR in complex with ZM241385 [66] had a more similar conformation for Tyr271 compared with the BPM-derived homology model, however.

This work has demonstrated how SPR can be used to screen a library of small-molecule ligands for binding to a real GPCR and how the resultant binding and kinetic data can be used to create a three-dimensional picture of the ligand-binding site.

4.1.7. β_1 -Adrenergic receptor

A biophysical fragment screening approach using SPR for the initial screen has recently been applied to the thermostabilised turkey β_1 -adrenergic receptor (β_1 AR) [67]. Alongside the thermostabilised

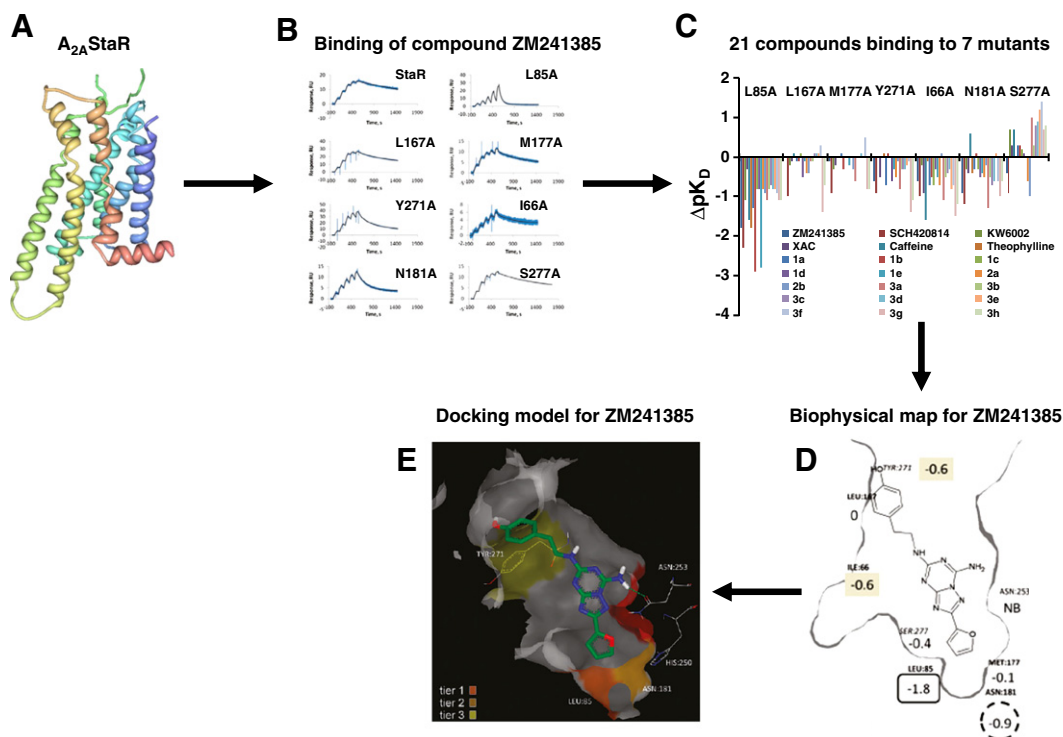


Fig. 4. Biophysical mapping of the adenosine A_{2A} receptor using SPR. A. The procedure starts with a stabilised receptor (StaR) minimally engineered for thermostability. B. Sensorgrams for binding of compound ZM241385 to StaR and mutant forms of A_{2A} . Following introduction of further single mutations at positions proposed to be in the ligand binding site, SPR measurements of ligand binding are performed on the StaR and mutant receptors. C. Matrix of SPR responses (as a log difference compared with unaltered StaR background) for 21 compounds binding to 7 different mutants of A_{2A} . D. Biophysical representation from the SPR data for compound ZM241385 based on a homology model of A_{2A} . Each of the shown residues was mutated to alanine and the log difference value for binding compound ZM241385 is shown. Key: residues in bold font = in front of the plane, italics = behind the plane, normal font = in the plane of the ligand, NB = non-binding, black oval = largest effect, dotted circle = second largest effect, shaded box = third largest effects. E. Docked structure of compound ZM241385 in a homology model of A_{2A} . Asn 253 is coloured red as mutation of this residue prevents binding of ligands. The first, second and third tier effects of mutations are coloured according to the key and relate to the residues indicated in D from the BPM data. (Picture A was produced using the PDB file (PDB ID: 3PWH) and PDB Protein Workshop 3.9 from Moreland et al. 2005; B was modified from Zhukov et al. [63]; C was constructed from data given in Zhukov et al. [63]; D and E were reproduced from Zhukov et al. [63]).

A_{2A} receptor, thermostabilised β_1 AR StaR was screened for binding a subset of the Heptares fragment library (~650 fragments) using SPR with the receptors immobilised on nickel-charged NTA sensor chips. Among the fragments that bound selectively to β_1 AR StaR were two arylpiperazine compounds with binding affinities (K_D values) of 16 and 5.6 μ M and good ligand efficiencies of 0.41 and 0.48 (Fig. 5A). A fragment hit-to-lead exercise was then performed using a radioligand binding assay, which identified a number of fragments that bound with even higher affinity including an indole compound and a quinoline compound (Fig. 5B). Crystal structures of thermostabilised β_1 AR

in complex with these two compounds were solved at resolutions of 2.8 and 2.7 Å, respectively. The observed protein–ligand interactions in the crystal structures suggested that these compounds are antagonists of β_1 AR. These results demonstrate a first full fragment-based drug discovery program applied to a GPCR with screening using SPR.

4.2. Non-GPCRs

In addition to GPCRs, SPR methods have been developed and used to characterise ligand binding with a range of other membrane protein systems, which are described below.

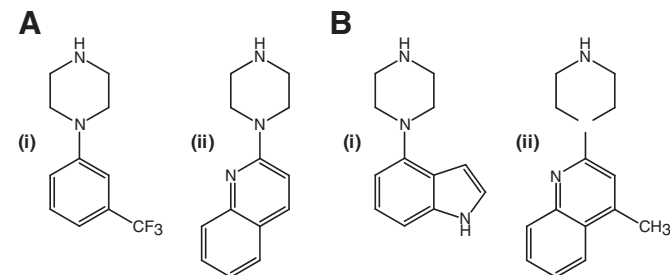


Fig. 5. High affinity ligands for the β_1 -adrenergic receptor identified by biophysical fragment screening. A. Arylpiperazine compounds with binding affinities (K_D values) of 16 and 5.6 μ M for (i) and (ii), respectively, identified by SPR screening of a fragment library against thermostabilised turkey β_1 AR. B. (i) Indole and (ii) Quinoline compounds identified as higher affinity ligands of β_1 AR from a fragment hit-to-lead exercise using a radioligand binding assay based on the compounds shown in A. (These chemical structures were taken from Christopher et al. [67]).

4.2.1. Outer membrane receptor FhuA

SPR has been used to probe for conformational changes in the FhuA outer membrane receptor of *E. coli*, which transports iron-chelating siderophores into the cytoplasm, by observing the binding of monoclonal antibodies [68]. These measurements were performed with FhuA in its apo- and siderophore-bound states with ferricrocin and in the absence and presence of protein TonB, which is found in the cytoplasmic membrane and transduces energy to FhuA to facilitate siderophore transport. Four monoclonal antibodies were produced that mapped to epitopes on outer surface-exposed loops 3, 4 and 5 and to β -barrel strand 14. For measurements of antibody binding to FhuA, the antibodies were immobilised separately on CM4 sensor chips using amine coupling chemistry and FhuA was injected in the analyte flow. For measurements involving TonB, this protein was immobilised on the sensor chip using thiol coupling chemistry followed by injection of FhuA and then the antibodies. SPR data was

used to measure the kinetics of all binding interactions, which revealed that binding of TonB to FhuA promotes conformational changes in outer surface-exposed loops 3 and 5 of FhuA. The data also suggested that the presence of ferricrocin alters the properties of the FhuA-TonB binding interaction and therefore influences the resultant conformational changes.

4.2.2. Tyrosine kinase HER2 receptor subdomain

Using the tyrosine kinase receptor HER2 as a proof of principle, a medium-throughput ligand screening strategy has been developed using synthetic peptides that mimic a selected subdomain of the target protein [69]. In this case, a modified fragment that mimics HER2 domain IV in its Herceptin-bound conformation was designed and immobilised using a biotinylated N-terminus on a streptavidin-coated sensor chip. Injection of the antibody Herceptin in the analyte flow at a range of concentrations produced SPR responses from which the measured binding affinity (K_D , 19.2 nM) and kinetic rate constants verified the approach for SPR analysis of ligand binding.

4.2.3. Human (pro)renin receptor

SPR has been used to investigate binding of human (pro)renin receptor to human renin with the receptor in three forms: full-length (hPRR), lacking the cytoplasmic domain (hPRR- Δ CD) and just the extracellular domain (hPRR- Δ TM Δ CD) [70]. Purified human renin was immobilised on a sensor chip using amine coupling chemistry then the three purified receptor forms injected in the analyte flow at a range of concentrations. The SPR data showed binding affinities for full-length hPRR and hPRR- Δ CD of 46 and 330 nM, respectively, suggesting that the cytoplasmic domain of hPRR is not essential for the binding of renin. The hPRR- Δ TM Δ CD form showed no binding affinity, therefore demonstrating that the purified hPRR extracellular domain does not have the ability to bind with human renin. Extracellular domain obtained from the microsomal fraction (non-purified) did retain full renin binding activity compared with full-length hPRR, however.

4.2.4. α -Hemolysin

Using α -hemolysin and binding of its specific antibody as a model system, a novel SPR approach using arrays of periodic nanopores in a free-standing metal film and pore-spanning lipid membranes has been developed for kinetic binding assays [71] (Fig. 6). This differs from conventional SPR since it is based on the phenomenon of an extraordinary optical transmission (EOT) effect [72] through periodic nanopore arrays in metallic films (Fig. 6A), in this case using an Au/Si₃N₄ film. The patterned nanopores in the metal film are encapsulated in a silica layer then a pore-spanning lipid membrane is formed

over the surface by vesicle rupture. Since part of the lipid membrane is suspended over the nanopores it is accessible from both sides and therefore better resembles a natural lipid membrane. A target protein can be reconstituted into the lipid membrane and the binding of ligands changes the local refractive index and the EOT effect through the nanopores. In the transmission spectra, the resonance wavelength red-shifted on forming the lipid membrane from phosphatidylcholine vesicles and then shifted further on incorporation of heptameric α -hemolysin into the lipid membrane and then further again on binding biotinylated anti- α -hemolysin antibody (Fig. 6B). Real-time kinetic measurements were made to follow these events and then to monitor the binding of a range in concentrations of the antibody (Fig. 6C) and the response used to measure a binding affinity (K_D) of 19 ± 10 nM. Binding of streptavidin-R-phycoerythrin to the antibody further confirmed the specific binding interaction of the antibody with α -hemolysin.

4.2.5. β -Site amyloid precursor protein cleaving enzyme 1

An SPR ligand binding assay for full-length β -site amyloid precursor protein cleaving enzyme 1 (BACE1) reconstituted in native brain lipid membranes has been developed [73]. This protein, which has a single transmembrane-spanning domain, is responsible for controlling the formation of peptides that are constituents of amyloid plaques, so it is therefore a drug target for Alzheimer's disease. BACE1 was expressed in insect cells and captured directly from the cell lysate on to an L1 sensor chip surface immobilised using amine coupling chemistry with an antibody specific for a His₆ tag. The protein was then reconstituted into a membrane formed from brain lipid extract and tested for the binding of six different known BACE1 inhibitors. This analysis was performed using two different pH values of 7.4 and 4.5 and in the presence of added calcium. Kinetic analysis of the SPR responses showed different binding characteristics for the different compounds and at the different pH values, the addition of calcium had no significant effects on these.

4.2.6. Human CD4 receptor in nanodiscs

Using the human CD4 receptor as a model system, a new SPR approach with membrane proteins reconstituted in nanodiscs as the analyte has been developed for ligand-binding studies [74]. Nanodiscs are discoidal model membrane systems that can encapsulate and solubilise integral membrane proteins in a near-native environment and have already been used with a number of biophysical techniques. This work used a cysteine replacement variant of the transmembrane and cytoplasmic domains (residues 372–433) of human CD4 fused to ubiquitin with a His₁₀ tag at its N-terminus referred to as His-Ubi-CD4. Nanodiscs containing this fusion protein were constructed using a membrane

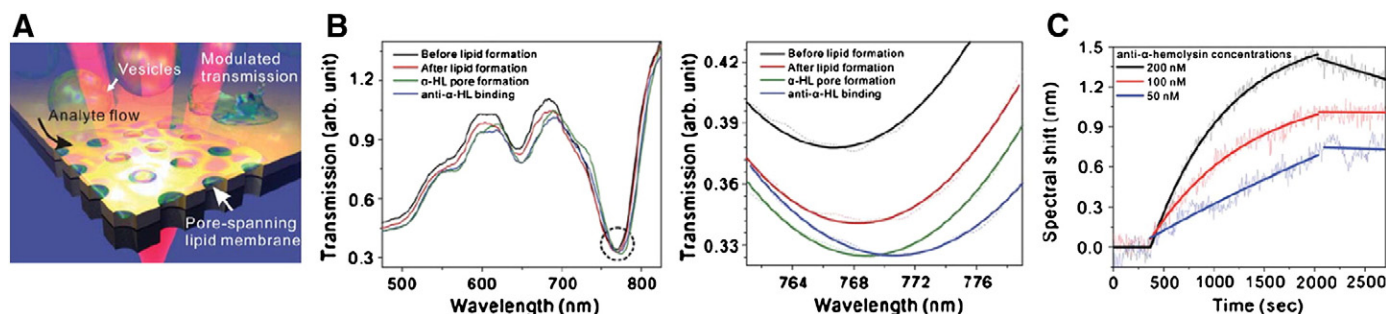


Fig. 6. Detection of antibody binding to α -hemolysin using a plasmonic nanopore array and pore-spanning lipid membrane. A. Cartoon representation of a nanopore array in a metal film with a pore-spanning lipid membrane. The transmission of light through the nanopores is modulated by the presence of a lipid membrane formed by vesicle rupture and subsequent binding of molecules. The lipid membrane is suspended over the nanopores such that it can be accessed from both sides and therefore better mimics a natural cell membrane. B. Transmission spectra change before (black line) and after formation of a pore-spanning lipid membrane (red line), after formation of a α -hemolysin pore on the lipid membrane (green line) and after binding of anti- α -hemolysin antibody (blue line) on a Au/Si₃N₄ film with a periodic array of nanopores. C. Real-time kinetic measurements for anti- α -hemolysin antibody binding at a range of concentrations to α -hemolysin in a suspended lipid membrane on a nanopore array. (Pictures A–C were reproduced from Im et al. [71]).

scaffold protein (MSP) and POPC lipids with isolation and purification by gel filtration chromatography and solutions of these were used as the analyte in the SPR measurements. The resultant nanodiscs contained one His-Ubi-CD4 molecule per nanodisc. For binding to a PentaHis monoclonal antibody immobilised on a CM5 sensor chip through amine coupling, control analyte solutions contained His-Ubi (not fused to CD4) or empty nanodiscs. Measurements with empty nanodiscs were subtracted from measurements with His-Ubi-CD4-nanodiscs to correct for any background non-specific binding. Kinetic analysis of SPR data obtained using a range in analyte concentrations gave affinities and rate constants in the expected range with K_D values of 10 and 11 nM for binding His-Ubi and His-Ubi-CD4-nanodiscs, respectively. This work demonstrated that it is feasible to use membrane proteins solubilised in nanodiscs as the analyte for SPR measurements of ligand binding.

4.2.7. Human ABC transporter P-gp in nanodiscs

SPR has been used to probe the conformation of human ATP-binding cassette transporter P-gp reconstituted in lipid nanodiscs and the binding of inhibitory antibodies [75]. P-gp mediates the efflux of drugs that contributes to cancer cell drug resistance, so is a target for new therapeutics that modulates the effectiveness of such drugs. The antibodies MRK16 and UIC2 were immobilised separately on CM5 sensor chips using amine-coupling chemistry. Purified P-gp reconstituted in MSP1D1 nanodiscs was injected in the analyte flow in the absence and presence of the drug vinblastine and the non-hydrolysable nucleotide AMPPNP and with ADP or ADP plus VO_4 . P-gp was shown to bind to both antibodies in the absence of drug and in the presence of AMPPNP or AMP. The affinity and kinetics for binding of the P-gp nanodiscs to the antibodies were not affected by the presence of vinblastine. The results also suggested that drugs are not released from the ADP- VO_4 -trapped state.

4.2.8. Epidermal growth factor receptor on intact cells

A novel intact-cell-based SPR method for measurements of ligand binding has been developed and demonstrated with the epidermal growth factor (EGF) receptor [76]. The peptide ligand EGF was biotinylated using a polyethylene glycol spacer by coupling with its amine groups and then immobilised on a streptavidin-coated sensor

chip. A suspension of human carcinoma A431 cells was injected in the analyte flow and the resultant SPR responses were consistent with binding to the immobilised EGF. The specificity of the interaction was confirmed by competitive reduction of this response by the free EGF ligand added at a range of concentrations in the analyte flow along with the cells.

4.2.9. β_3 γ -aminobutyric acid type A receptors

An SPR assay has been used to screen the binding of 51 histaminergic and 15 GABAergic ligands with full-length homo-oligomeric β_3 γ -aminobutyric acid type A ($GABA_A$) receptors [77] (Fig. 7), which belong to the superfamily of Cys-loop ligand-gated ion channels and are involved in a wide range of neurological functions. Though the homo-oligomeric forms of these receptors have not yet been identified in the human brain, they serve as useful model systems for investigating receptor function and pharmacology [77]. This work used rat homo-oligomeric β_3 $GABA_A$ receptors with a His₈-tag that were expressed in insect cells, purified from isolated membranes and solubilised in detergent. The receptors were captured by polyhistidine monoclonal antibodies that were immobilised on CM3 and CM5 sensor chip surfaces using amine coupling chemistry, a control flow cell had a surface with immobilised antibodies without bound receptors. Ligands were injected at a range of concentrations with a 2-fold dilution series in the analyte flow. Equilibrium dissociation constants (K_D) were determined by non-linear regression analysis of steady-state SPR signals as a function of ligand concentration using a Langmuir isotherm equation. In addition to direct interaction binding of ligands with the receptors, competitive binding with histamine was also measured for a more rigorous analysis. Of the 51 histaminergic ligands tested, 17 had a binding interaction with a K_D value of less than 300 μ M (Fig. 7). Despite its small size, binding of histamine could be detected giving a K_D value of 100 μ M, which is in the same order of magnitude as values obtained from electrophysiological measurements on human homo-oligomeric receptors [77]. Histamine H1 receptor ligands did not interact with the β_3 receptors, binding of histamine H2 receptor agonists, except histamine, was not detected, whilst three histamine H2 receptor antagonists bound with a higher affinity than histamine (tiotidine, burimamide and famotidine). Some histamine H3/H4 receptor ligands showed binding to the β_3 receptors, five with a higher affinity than histamine (agonists (S)- α -methylhistamine,

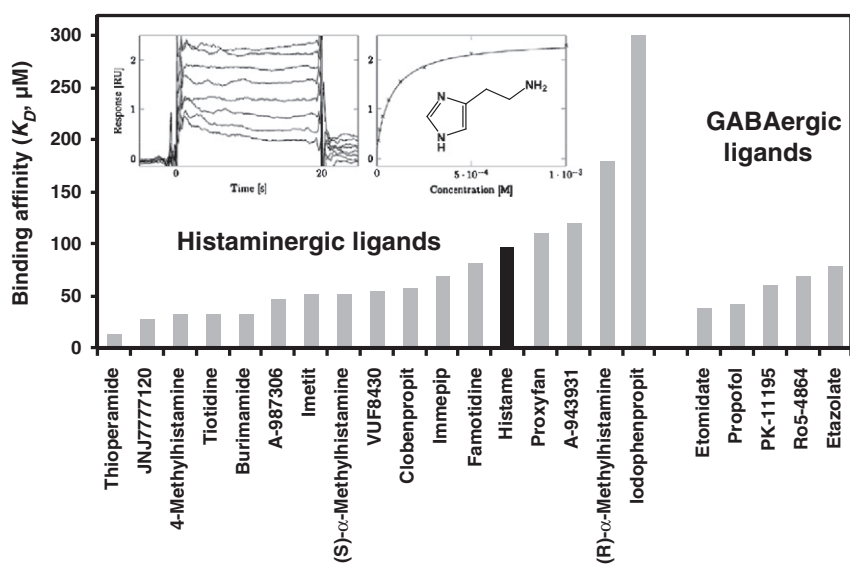


Fig. 7. Screening of ligand binding by full-length homo-oligomeric β_3 γ -aminobutyric acid type A ($GABA_A$) receptors by SPR. A. Bar-chart of binding affinities for histaminergic and GABAergic ligands that showed a binding effect when injected at a range of concentrations in the analyte flow over full-length homo-oligomeric β_3 γ -aminobutyric acid type A ($GABA_A$) receptors captured on a sensor chip. Inset are the sensorgrams for binding of histamine injected in a 2-fold dilution series from 1000 to 8 μ M (left) and the steady state signals plotted as a function of concentration with a fitted Langmuir binding isotherm, which gave a binding affinity (K_D) of 98 μ M (right). The structure of histamine is also shown. (The bar-chart was constructed from data given in Seeger et al. [77]; the sensorgrams and binding curve were reproduced from Seeger et al. [77]).

imetit and imnepip and antagonists thioperamide and clobenpropit). Some histamine H4 receptor ligands also bound to the receptors with a higher affinity than histamine, including 4-methylhistamine (Fig. 7). Of the 15 GABAergic ligands tested, five known active compounds showed a binding interaction with the β_3 receptors of higher affinity than that of histamine, but still in the low micromolar range (Fig. 7), whilst the others showed no binding up to a concentration of 100 μM . In the competition measurements, thirteen of the active histaminergic ligands competed with histamine whilst none of the GABAergic ligands showed a competitive effect. This work not only confirmed that GABA_A receptors have distinct histaminergic pharmacology in agreement with previous results, it also identified new ligands of the β_3 receptor. It is noteworthy that 200 ligand injections on a single sensor surface in ~ 20 h was possible; this is sufficiently medium-throughput to enable screening for higher affinity ligands with potential as histaminergic drugs by fragment-based drug discovery.

5. Recent developments and potential for drug discovery

Next-generation SPR instruments use a sensor surface based on nano-structured materials [78] (Figs. 6 and 8A). Unlike the BIAcore™ technology, which uses a prism to focus the light, these instruments are based on the phenomenon of extraordinary optical transmission (EOT) [72] where light at specific wavelengths transmitted through nano-holes in thin metal films is of higher intensity than the incident light. This is a consequence of plasmon generation in the metal film.

When a large number of nanopores are arranged in a periodic array in a metal film, their combined plasmon generation ‘funnels’ the light energy across the film. When molecules bind to the metal surface the specific wavelength of light for optimum transmission is shifted, so the surface can be used as a sensor. Due to the large number of nanopores that can be patterned in to the metal film, the sensing capability of this approach is much greater than can be achieved by a conventional SPR instrument. Furthermore, a lipid bilayer can be suspended above the nanopores and contain a membrane protein of interest. Since the bilayer can be accessed from both sides of the pore, this now allows SPR analysis of ligand binding to membrane proteins in a more native or near-native environment. This type of approach was demonstrated with α -hemolysin binding to its specific antibody described earlier [71].

An exciting new technique called surface plasmon resonance microscopy (SPRM) has recently been demonstrated that enables measurement of binding kinetics of membrane proteins in single living cells and therefore in their true native membrane environment [79,80] (Fig. 8B). The technique also allows the simultaneous measurement of optical and fluorescence imaging of the same sample. Cells are cultured on a gold-coated slide and SPRM imaging is performed using an inverted microscope. Binding of ligands to receptor proteins on the cell surface can be monitored by SPRM with millisecond temporal and micrometer spatial resolution. So far this technique has been used to measure the binding interaction between glycoproteins on the cell surface and lectin injected as analyte and the binding activity and spatial distribution of nicotinic acetylcholine

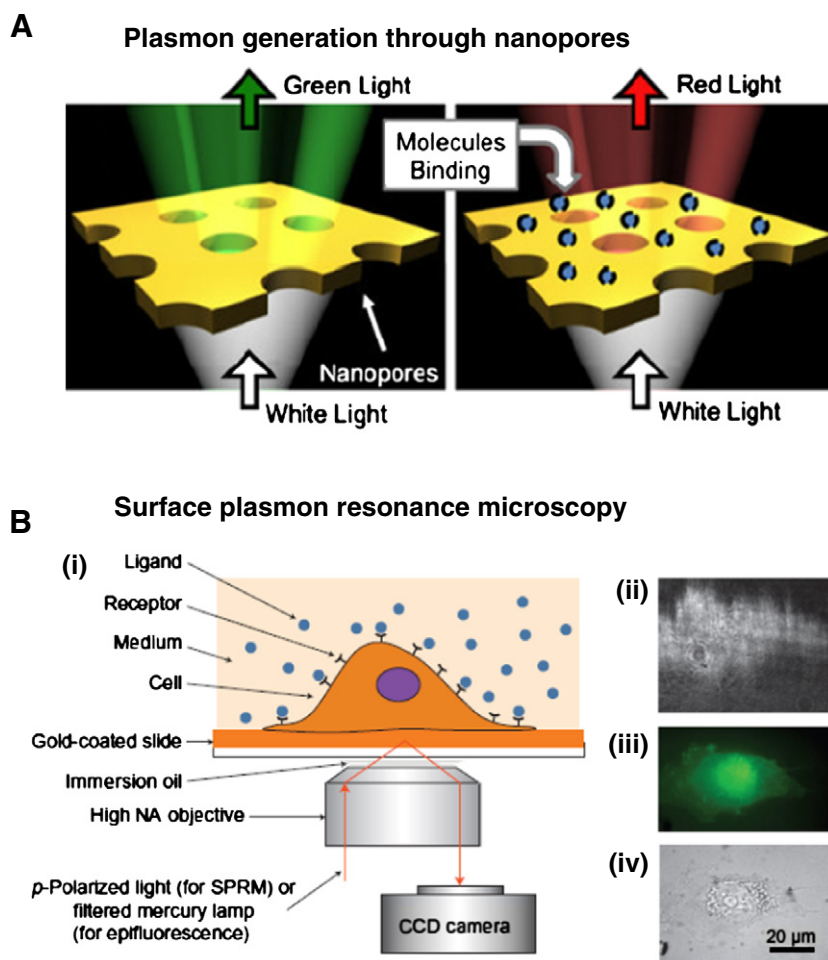


Fig. 8. Next-generation SPR instrumentation for measuring membrane protein–ligand binding. A. Nanopores in a gold film through which there is enhanced transmission of light due to plasmon generation which undergoes a red-shift on binding of molecules. B. Surface plasmon resonance microscopy with intact living cells. (i) Schematic illustration of the experimental set-up; (ii) SPR image of a cell; (iii) fluorescence image of a cell; (iv) bright-field image of a cell. (Picture A was modified from Maynard et al. [78] and B was modified from Wang et al. [80]).

receptors. Furthermore, SPRM allows simultaneous measurement of binding kinetics from thousands of sample spots, thus providing a significant enhancement in sensitivity over conventional SPR.

The important drug discovery method of fragment-based screening has successfully been combined with SPR for the medium-throughput screening of chemical libraries [81–87]. So far this has mostly been demonstrated with soluble non-membrane protein targets, but there is clearly high potential for combining fragment-based drug screening with the SPR technological advances already described and membrane protein targets, recently exemplified by results demonstrated with the β_1 -adrenergic receptor (Section 4.1.7).

6. Conclusions

This review has demonstrated that SPR is a rapidly developing technique for the quantitative characterisation of real-time binding and kinetics of membrane protein–ligand interactions that is label-free and uses relatively small quantities of materials. It can be used with a wide range of membrane protein systems including GPCRs, which are the major molecular targets for current validated drugs and for foreseeable drug discovery. Recent developments in SPR instrumentation, sensor chip design, sample preparation strategies and the increasing availability of cloned, stabilised and purified eukaryotic membrane proteins shows high potential for medium-throughput screening of libraries in the search for new small-molecule and monoclonal antibody drugs.

Acknowledgements

This work was funded by the European Drug Initiative for Channels and Transporters consortium (EDICT, contract 201924).

References

- [1] E. Wallin, G. von Heijne, Genome-wide analysis of integral membrane proteins from eubacterial, archaean, and eukaryotic organisms, *Prot. Sci.* 7 (1998) 1029–1038.
- [2] J. Liu, B. Rost, Comparing function and structure between entire proteomes, *Prot. Sci.* 10 (2001) 1970–1979.
- [3] L. Fagerberg, K. Jonasson, G. von Heijne, M. Uhlén, L. Berglund, Prediction of the human membrane proteome, *Proteomics* 10 (2010) 1141–1149.
- [4] A.W. Partridge, A.G. Therien, C.M. Deber, Polar mutations in membrane proteins as a biophysical basis for disease, *Biopolymers* 66 (2002) 350–358.
- [5] C.R. Sanders, J.K. Myers, Disease-related misassembly of membrane proteins, *Annu. Rev. Biophys. Biomol. Struct.* 33 (2004) 25–51.
- [6] G. von Heijne, The membrane protein universe: what's out there and why bother? *J. Int. Med.* 261 (2007) 543–557.
- [7] H. Watanabe, T.T. Koopmann, S.L. Scouarnec, T. Yang, C.R. Ingram, J.-J. Schott, S. Demolombe, V. Probst, F. Anselme, D. Escande, A.C.P. Wiesfeld, A. Pfeuffer, S. Kääh, H.-E. Wichmann, C. Hasdemir, Y. Aizawa, A.A.M. Wilde, D.M. Roden, C.R. Bezzina, Sodium channel β_1 subunit mutations associated with Brugada syndrome and cardiac conduction disease in humans, *J. Clin. Invest.* 118 (2008) 2260–2268.
- [8] D.M. Rosenbaum, S.G.F. Rasmussen, B.K. Kobilka, The structure and function of G-protein-coupled receptors, *Nature* 459 (2009) 356–363.
- [9] A.K. Kurze, G. Galliciotti, C. Heine, S.E. Mole, A. Quitsch, T. Braulke, Pathogenic mutations cause rapid degradation of lysosomal storage disease-related membrane protein CLN6, *Hum. Mutat.* 31 (2010) 1163–1174.
- [10] H.D. Shukla, P. Vaitiekunas, R.J. Cotter, Advances in membrane proteomics and cancer biomarker discovery: current status and future perspective, *Proteomics* 12 (2012) 3085–3104.
- [11] J. Drews, Drug discovery: a historical perspective, *Science* 287 (2000) 1960–1964.
- [12] A.L. Hopkins, C.R. Groom, The druggable genome, *Nat. Rev. Drug Discov.* 1 (2002) 727–730.
- [13] J.P. Overington, B. Al-Lazikani, A.L. Hopkins, Opinion—how many drug targets are there? *Nat. Rev. Drug Discov.* 5 (2006) 993–996.
- [14] K. Lundstrom, Latest development in drug discovery on G protein-coupled receptors, *Curr. Protein Pept. Sci.* 7 (2006) 465–470.
- [15] T.M. Bakheet, A.J. Doig, Properties and identification of human protein drug targets, *Bioinformatics* 25 (2009) 451–457.
- [16] I. Bahar, T.R. Lezon, A. Bakan, I.H. Shrivastava, Normal mode analysis of biomolecular structures: functional mechanisms of membrane proteins, *Chem. Rev.* 110 (2010) 1463–1497.
- [17] M. Rask-Andersen, M. Sällman Almén, H.B. Schiöth, Trends in the exploitation of novel drug targets, *Nat. Rev. Drug Discov.* 10 (2011) 579–590.
- [18] <http://www.rcsb.org/pdb/home/home.do>.
- [19] <http://blanco.biomol.uci.edu/mpstruc/listAll/list>.
- [20] M.A. Cooper, Advances in membrane receptor screening and analysis, *J. Mol. Recognit.* 17 (2004) 286–315.
- [21] M. Besenica, P. Macek, J.H. Lakey, G. Anderluh, Surface plasmon resonance in protein–membrane interactions, *Chem. Phys. Lipids* 141 (2006) 169–178.
- [22] Z. Salamon, H.A. Macleod, G. Tollin, Surface plasmon resonance spectroscopy as a tool for investigating the biochemical and biophysical properties of membrane protein systems. I: Theoretical principles, *Biochim. Biophys. Acta—Reviews on Biomembranes* 1331 (1997) 117–129.
- [23] K. Kurihara, K. Suzuki, Theoretical understanding of an absorption-based surface plasmon resonance sensor based on Kretschmann's theory, *Anal. Chem.* 74 (2002) 696–701.
- [24] J. Homola, Present and future of surface plasmon resonance biosensors, *Anal. Bioanal. Chem.* 377 (2003) 528–539.
- [25] K.S. Phillips, Q. Cheng, Recent advances in surface plasmon resonance based techniques for bioanalysis, *Anal. Bioanal. Chem.* 387 (2007) 1831–1840.
- [26] M. Piliarik, H. Vaisocherová, J. Homola, Surface plasmon resonance biosensing, *Methods Mol. Biol.* 503 (2009) 65–88.
- [27] H.N. Daghestani, Theory and applications of surface plasmon resonance, resonant mirror, resonant waveguide grating, and dual polarization interferometry biosensors, *Sensors (Basel)* 10 (2010) 9630–9646.
- [28] M. Raghavan, P.J. Bjorkman, BIACore: a microchip-based system for analyzing the formation of macromolecular complexes, *Structure* 3 (1995) 331–333.
- [29] L. Nieba, S.E. Nieba-Axmann, A. Persson, M. Hämäläinen, F. Edebratt, A. Hansson, J. Lidholm, K. Magnusson, A.F. Karlsson, A. Plückthun, BIACORE analysis of histidine-tagged proteins using a chelating NTA sensor chip, *Anal. Biochem.* 252 (1997) 217–228.
- [30] M. Malmqvist, BIACORE: an affinity biosensor system for characterization of biomolecular interactions, *Biochem. Soc. Trans.* 27 (1999) 335–340.
- [31] D.G. Myszka, Kinetic, equilibrium, and thermodynamic analysis of macromolecular interactions with BIACORE, *Methods Enzymol.* 323 (2000) 325–340.
- [32] R.L. Rich, D.G. Myszka, BIACORE J: a new platform for routine biomolecular interaction analysis, *J. Mol. Recognit.* 14 (2001) 223–228.
- [33] I. Navratilova, E. Eisenstien, D.G. Myszka, Measuring long association phases using Biacore, *Anal. Biochem.* 344 (2005) 295–297.
- [34] L. Jason-Moller, M. Murphy, J. Bruno, Overview of Biacore systems and their applications, *Curr. Protoc. Protein Sci.* 45 (2006) 19.13.1–19.13.14.
- [35] R.L. Rich, J. Errey, F. Marshall, D.G. Myszka, Biacore analysis with stabilized G-protein-coupled receptors, *Anal. Biochem.* 409 (2011) 267–272.
- [36] P. Leonard, S. Hearty, R. O'Kennedy, Measuring protein–protein interactions using Biacore, *Methods Mol. Biol.* 681 (2011) 403–418.
- [37] Z. Altintas, Y. Uludag, Y. Gurbuz, I. Tothill, Development of surface chemistry for SPR based sensors for the detection of proteins and DNA molecules, *Anal. Chim. Acta* 712 (2012) 138–144.
- [38] BIACORE AB, BIACORE Technology Handbook, 1998.
- [39] M.A. Cooper, A. Hansson, S. Löfås, D.H. Williams, A vesicle capture sensor chip for kinetic analysis of interactions with membrane-bound receptors, *Anal. Biochem.* 277 (2000) 196–205.
- [40] H. Mozsolits, W.G. Thomas, M.I. Aguilar, Surface plasmon resonance spectroscopy in the study of membrane-mediated cell signalling, *J. Pept. Sci.* 9 (2003) 77–89.
- [41] Y.J. Li, L.J. Bi, X.E. Zhang, Y.F. Zhou, J.B. Zhang, Y.Y. Chen, W. Li, Z.P. Zhang, Reversible immobilization of proteins with streptavidin affinity tags on a surface plasmon resonance biosensor chip, *Anal. Bioanal. Chem.* 386 (2006) 1321–1326.
- [42] G. Papalia, D. Myszka, Exploring minimal biotinylation conditions for biosensor analysis using capture chips, *Anal. Biochem.* 403 (2010) 30–35.
- [43] Z. Salamon, Y. Wang, J.L. Soulages, M.F. Brown, G. Tollin, Surface plasmon resonance spectroscopy studies of membrane proteins: transduction binding and activation by rhodopsin monitored in thin membrane films, *Biophys. J.* 71 (1996) 283–294.
- [44] S. Heyse, O.P. Ernst, Z. Dienes, K.P. Hofmann, H. Vogel, Incorporation of rhodopsin in laterally structured supported membranes: observation of transducin activation with spatially and time-resolved surface plasmon resonance, *Biochemistry* 37 (1998) 507–522.
- [45] C. Bieri, O.P. Ernst, S. Heyse, K.P. Hofmann, H. Vogel, Micropatterned immobilization of a G protein-coupled receptor and direct detection of G protein activation, *Nat. Biotechnol.* 17 (1999) 1105–1108.
- [46] E. Sackmann, Supported membranes: scientific and practical applications, *Science* 271 (1996) 43–48.
- [47] H. Lang, C. Duschl, H. Vogel, A new class of thiolipids for the attachment of lipid bilayers on gold surfaces, *Langmuir* 10 (1994) 197–210.
- [48] O.P. Karlsson, S. Löfås, Flow-mediated on-surface reconstitution of G-protein coupled receptors for applications in surface plasmon resonance biosensors, *Anal. Biochem.* 300 (2002) 132–138.
- [49] F. Hüttenrauch, A. Nitzki, F.T. Lin, S. Höning, M. Oppermann, Beta-arrestin binding to CC chemokine receptor 5 requires multiple C-terminal receptor phosphorylation sites and involves a conserved Asp-Arg-Tyr sequence motif, *J. Biol. Chem.* 277 (2002) 30769–30777.
- [50] P. Stenlund, G.J. Babcock, J. Sodroski, D.G. Myszka, Capture and reconstitution of G protein-coupled receptors on a biosensor surface, *Anal. Biochem.* 316 (2003) 243–250.
- [51] I. Navratilova, J. Sodroski, D.G. Myszka, Solubilisation, stabilisation, and purification of chemokine receptors using biosensor technology, *Anal. Biochem.* 339 (2005) 271–281.
- [52] I. Navratilova, M. Dioszegi, D.G. Myszka, Analyzing ligand and small molecule binding activity of solubilised GPCRs using biosensor technology, *Anal. Biochem.* 355 (2006) 132–139.

- [53] V.I. Silin, E.A. Karlik, K.D. Ridge, D.J. Vanderah, Development of surface-based assays for transmembrane proteins: selective immobilization of functional CCR5, a G protein-coupled receptor, *Anal. Biochem.* 349 (2006) 247–253.
- [54] I. Navratilova, M. Pancera, R.T. Wyatt, D.G. Myszka, A biosensor-based approach toward purification and crystallization of G protein-coupled receptors, *Anal. Biochem.* 353 (2006) 278–283.
- [55] R.L. Rich, A.R. Miles, B.K. Gale, D.G. Myszka, Detergent screening of a G-protein-coupled receptor using serial and array biosensor technologies, *Anal. Biochem.* 386 (2009) 98–104.
- [56] R. Mansfield, S. Able, P. Griffin, B. Irvine, I. James, M. Macartney, K. Miller, J. Mills, C. Napier, I. Navratilova, M. Perros, G. Rickett, H. Root, E. van der Ryst, M. Westby, P. Dorr, CCR5 pharmacology methodologies and associated applications, *Methods Enzymol.* 460 (2009) 17–55.
- [57] I. Navratilova, J. Besnard, A.L. Hopkins, Screening for GPCR ligands using surface plasmon resonance, *ACS Med. Chem. Lett.* 2 (2011) 549–554.
- [58] F. Alonzo III, L. Kozhaya, S.A. Rawlings, T. Reyes-Robles, A.L. DuMont, D.G. Myszka, N.R. Landau, D. Unutmaz, V.J. Torres, CCR5 is a receptor for *Staphylococcus aureus* leukotoxin ED, *Nature* 493 (2013) 51–55.
- [59] P.J. Harding, T.C. Hadingham, J.M. McDonnell, A. Watts, Direct analysis of a GPCR-agonist interaction by surface plasmon resonance, *Eur. Biophys. J.* 35 (2006) 709–712.
- [60] P.J. Harding, H. Attrill, S. Ross, J.R. Koeppe, A.N. Kapanidis, A. Watts, Neurotensin receptor type 1: *Escherichia coli* expression, purification, characterization and biophysical study, *Biochem. Soc. Trans.* 35 (2007) 760–763.
- [61] L. Kaiser, J. Graveland-Bikker, D. Steuerwald, M. Vanberghem, K. Herlihy, S. Zhang, Efficient cell-free production of olfactory receptors: detergent optimization, structure, and ligand binding analyses, *Proc. Natl. Acad. Sci. USA* 105 (2008) 15726–15731.
- [62] C. Zou, S. Kumaran, S. Markovic, R. Walser, O. Zerbe, Studies of the structure of the N-terminal domain from the Y4 receptor—a G protein-coupled receptor—and its interaction with hormones from the NPY family, *ChemBiochem* 9 (2008) 2276–2284.
- [63] A. Zhukov, S.P. Andrews, J.C. Errey, N. Robertson, B. Tehan, J.S. Mason, F.H. Marshall, M. Weir, M. Congreve, Biophysical mapping of the adenosine A_{2A} receptor, *J. Med. Chem.* 54 (2011) 4312–4323.
- [64] M. Congreve, R.L. Rich, D.G. Myszka, F. Figaroa, G. Siegal, F.H. Marshall, Fragment screening of stabilized G-protein-coupled receptors using biophysical methods, *Methods Enzymol.* 493 (2011) 115–136.
- [65] V.P. Jaakola, M.T. Griffith, M.A. Hanson, V. Cherezov, E.Y. Chien, J.R. Lane, A.P. Ijzerman, R.C. Stevens, The 2.6 angstrom crystal structure of a human A_{2A} adenosine receptor bound to an antagonist, *Science* 322 (2008) 1211–1217.
- [66] A.S. Doré, N. Robertson, J.C. Errey, I. Ng, K. Hollenstein, B. Tehan, E. Hurrell, K. Bennett, M. Congreve, F. Magnani, C.G. Tate, M. Weir, F.H. Marshall, Structure of the adenosine A_{2A} receptor in complex with ZM241385 and the xanthines XAC and caffeine, *Structure* 19 (2011) 1283–1293.
- [67] J.A. Christopher, J. Brown, A.S. Doré, J.C. Errey, M. Koglin, F.H. Marshall, D.G. Myszka, R.L. Rich, C.G. Tate, B. Tehan, T. Warne, M. Congreve, Biophysical fragment screening of the β 1-adrenergic receptor: identification of high affinity arylpiperazine leads using structure-based drug design, *J. Med. Chem.* 56 (2013) 3446–3455.
- [68] K.J. James, M.A. Hancock, V. Moreau, F. Molina, J.W. Coulton, TonB induces conformational changes in surface-exposed loops of FhuA, outer membrane receptor of *Escherichia coli*, *Protein Sci.* 17 (2008) 1679–1688.
- [69] L. Monfregola, R.M. Vitale, P. Amodeo, S. De Luca, A SPR strategy for high-throughput ligand screenings based on synthetic peptides mimicking a selected subdomain of the target protein: a proof of concept on HER2 receptor, *Bioorg. Med. Chem.* 17 (2009) 7015–7020.
- [70] D. Du, T. Kato, F. Suzuki, E.Y. Park, Binding affinity of full-length and extracellular domains of recombinant human (pro)renin receptor to human renin when expressed in the fat body and hemolymph of silkworm larvae, *J. Biosci. Bioeng.* 108 (2009) 304–309.
- [71] H. Im, N.J. Wittenberg, A. Lesuffleur, N.C. Lindquist, S.H. Oh, Membrane protein biosensing with plasmonic nanopore arrays and pore-spanning lipid membranes, *Chem. Sci.* 1 (2010) 688–696.
- [72] R. Gordon, D. Sinton, K.L. Kavanagh, A.G. Brolo, A new generation of sensors based on extraordinary optical transmission, *Acc. Chem. Res.* 41 (2008) 1049–1057.
- [73] T. Christopheit, G. Stenberg, T. Gossas, S. Nyström, V. Baraznenok, E. Lindström, U.H. Danielson, A surface plasmon resonance-based biosensor with full-length BACE1 in a reconstituted membrane, *Anal. Biochem.* 414 (2011) 14–22.
- [74] J.M. Glück, B.W. Koenig, D. Willbold, Nanodiscs allow the use of integral membrane proteins as analytes in surface plasmon resonance studies, *Anal. Biochem.* 408 (2011) 46–52.
- [75] T.K. Ritchie, H. Kwon, W.M. Atkins, Conformational analysis of human ATP-binding cassette transporter ABCB1 in lipid nanodiscs and inhibition by the antibodies MRK16 and UIC2, *J. Biol. Chem.* 286 (2011) 39489–39496.
- [76] T. Mizuguchi, H. Uchimura, H. Kataoka, K. Akaji, Y. Kiso, K. Saito, Intact-cell-based surface plasmon resonance measurements for ligand affinity evaluation of a membrane receptor, *Anal. Biochem.* 420 (2012) 185–187.
- [77] C. Seeger, T. Christopheit, K. Fuchs, K. Grote, W. Sieghart, U.H. Danielson, Histaminergic pharmacology of homo-oligomeric β 3 γ -aminobutyric acid type A receptors characterized by surface plasmon resonance biosensor technology, *Biochem. Pharmacol.* 84 (2012) 341–351.
- [78] J.A. Maynard, N.C. Lindquist, J.N. Sutherland, A. Lesuffleur, A.E. Warrington, M. Rodriguez, S.-H. Oh, Next generation SPR technology of membrane-bound proteins for ligand screening and biomarker discovery, *Biotechnol. J.* 4 (2009) 1542–1558.
- [79] C.T. Campbell, G. Kim, SPR microscopy and its applications to high-throughput analyses of biomolecular binding events and their kinetics, *Biomaterials* 28 (2007) 2380–2392.
- [80] W. Wang, Y. Yang, S. Wang, V.J. Nagaraj, Q. Liu, J. Wu, N. Tao, Label-free measuring and mapping of binding kinetics of membrane proteins in single living cells, *Nat. Chem.* 4 (2012) 846–853.
- [81] T. Neumann, H.D. Junker, K. Schmidt, R. Sekul, SPR-based fragment screening: advantages and applications, *Curr. Top. Med. Chem.* 7 (2007) 1630–1642.
- [82] S. Perspicace, D. Banner, J. Benz, F. Müller, D. Schlatter, W. Huber, Fragment-based screening using surface plasmon resonance technology, *J. Biomol. Screen.* 14 (2009) 337–349.
- [83] I. Navratilova, A.L. Hopkins, Fragment screening by surface plasmon resonance, *ACS Med. Chem. Lett.* 1 (2010) 44–48.
- [84] R.L. Rich, D.G. Myszka, Kinetic analysis and fragment screening with Fujifilm AP-3000, *Anal. Biochem.* 402 (2010) 170–178.
- [85] R.L. Rich, J.G. Quinn, T. Morton, J.D. Stepp, D.G. Myszka, Biosensor-based fragment screening using FastStep injections, *Anal. Biochem.* 407 (2010) 270–277.
- [86] T. Neumann, R. Sekul, SPR screening of chemical microarrays for fragment-based discovery, in: M. Cooper, L.M. Mayr (Eds.), *Label-free technologies for drug discovery*, John Wiley & Sons, Ltd, Chichester, UK, 2011, pp. 41–55.
- [87] M. Elinder, M. Geitmann, T. Gossas, P. Källblad, J. Winqvist, H. Nordström, M. Hämäläinen, U.H. Danielson, Experimental validation of a fragment library for lead discovery using SPR biosensor technology, *J. Biomol. Screen.* 16 (2011) 15–25.