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Plasmon Resonance Methods in GPCR Signaling and Other Membrane Events

I.D. Alves[§], C.K. Park[§], and V.J. Hruby^{§,†,*}

[§] *Department of Chemistry,*

[†] *Department of Biochemistry and Molecular Biophysics, University of Arizona, 85721 Tucson, Arizona, USA*

Abstract

The existence of surface guided electromagnetic waves has been theoretically predicted from Maxwell's equations and investigated during the first decades of the 20th century. However, it is only since the late 1960's that they have attracted the interest of surface physicists and earned the moniker of "surface plasmon". With the advent of commercially available instruments and well established theories, the technique has been used to study a wide variety of biochemical and biotechnological phenomena. Spectral response of the resonance condition serves as a sensitive indicator of the optical properties of thin films immobilized within a wavelength of the surface. This enhanced surface sensitivity has provided a boon to the surface sciences, and fosters collaboration between surface chemistry, physics and the ongoing biological and biotechnological revolution. Since then, techniques based on surface plasmons such as Surface Plasmon Resonance (SPR), SPR Imaging, Plasmon Waveguide Resonance (PWR) and others, have been increasingly used to determine the affinity and kinetics of a wide variety of real time molecular interactions such as protein-protein, lipid-protein and ligand-protein, without the need for a molecular tag or label. The physical-chemical methodologies used to immobilize membranes at the surface of these optical devices are reviewed, pointing out advantages and limitations of each method. The paper serves to summarize both historical and more recent developments of these technologies for investigating structure-function aspects of these molecular interactions, and regulation of specific events in signal transduction by G-protein coupled receptors (GPCRs).

Keywords

Surface plasmon resonance; surface plasmon resonance imaging; plasmon waveguide resonance; G-protein coupled receptors; human delta opioid receptor; rhodopsin; signal transduction; thin films

ABBREVIATIONS

EM = Electromagnetic; PWR = Plasmon waveguide resonance; GPCR = G-protein coupled receptor; SAM = Self-assembled monolayer; GTP = Guanosine triphosphate; λ = Wavelength; α = Angle of incidence; SPR = Surface plasmon resonance; ATR = Attenuated total reflection; TIRF = Total internal reflectance fluorescence; MS = Mass spectrometry; SP = Surface plasmon; n = Refractive index; k = Extinction coefficient

*Address correspondence to this author at the 1306 E University Blvd., Old Chemistry Building, University of Arizona, 85721 Tucson, AZ, USA; Tel: (520) 621-6332; Fax: (520) 621-8407; E-mail:hruby@u.arizona.edu.

INTRODUCTION

The phenomenon of anomalous dispersion on diffraction gratings due to the excitation of surface plasma waves was first described in the beginning of the twentieth century. In the late 1960s, optical excitation of surface plasmons by the method of attenuated total reflection (ATR) was demonstrated by Kretschmann [1] and Otto [2]. The potential of SPR for characterization of thin films and monitoring processes at metal interfaces was recognized in the late seventies [3]. In 1982 the use of SPR for biosensing was demonstrated by Nylander and Liedberg [4]. Since then, SPR has been intensively studied [5,6] and found to be in good agreement with theoretical concepts based on Maxwell's theory of electromagnetism (EM).

With the development of the first commercial optical sensor in the late 80's the use of optical biosensors in academic research, in industrial research and in technology development has exploded, and more than 3,000 scientific publications have appeared covering a very broad range of disciplines. Applications include ligand screening, immunology, cell biology, signal transduction, and nucleotide-nucleotide, nucleotide-protein, protein-protein and protein-lipid interactions.

Recently, plasmon resonance-based techniques have been applied to the investigation of G-Protein Coupled Receptors (GPCRs). This superfamily is one of the largest families of proteins in mammals for which over 800 encoding sequences have been identified in the human genome [7]. More than 50% of the drugs on the market are their surrogate activators or inhibitors. Thus, an understanding of GPCRs signal transduction and mechanisms of ligand-receptor interactions have become central to understanding many aspects of health and disease. Despite their crucial importance, however, very little is known about membrane protein structure/function relationships, and their high-resolution structural analysis has been hindered, mainly as a consequence of their low natural abundance and the difficulty in producing, purifying, and crystallizing significant quantities of tissue-derived or recombinant protein derived receptor. Techniques requiring small quantities of material, allowing non-invasive studies, while keeping those proteins in a native-like environment are ideal for their investigation. Plasmon resonance techniques, being direct and highly sensitive methods, offer the possibility for the protein to be in a lipid bilayer-like environment, and constitute ideal methods for investigating the various aspects of protein-ligand, protein-protein and protein-lipid interactions occurring in GPCR signaling in the kinetic and equilibrium modes.

In this review, plasmon resonance techniques such as SPR, SPR-imaging, SPR-MS (mass spectrometry) and PWR will be reviewed in terms of their fundamentals, and in the latest applications with emphasis in studies performed with membrane proteins. Covalent and non-covalent immobilization methods for the attachment of the molecules under study to sensor surfaces are reviewed as well as methods for the functional reconstitution of membrane proteins in the various types of lipid mono/bilayers. Advantages and disadvantages of each methodology are provided along with some of the latest accomplishments with emphasis on the area of GPCRs.

Surface Plasmon Resonance (SPR)

In Maxwell's theory of electromagnetism, plasmons are treated as charge density oscillations of the free electrons of a metal. These electron density fluctuations generate a surface localized electromagnetic (EM) wave which nonradiatively propagates along a metal/dielectric interface. The electric field is normal to this interface and vanishes exponentially with distance. The EM characteristics are the same as those describing optically generated evanescent waves in total internal reflection techniques. The EM fields do not stop at the boundary, but penetrate a distance into the emerging medium in the form of a surface wave.

Maxwell's equations have solutions resulting in SP waves only when two conditions are met. First, the media which generates plasmons must have a negative value for the real part of its complex dielectric constant, $\epsilon' = n^2 - k^2$. Most materials cannot be utilized as surface active media due to this first condition. Gold, silver and aluminum [8], are the best examples of materials which can support surface plasmons ($\epsilon' = -11, -15, -40$ respectively). The EM wave in the surface active medium is evanescent under all circumstances.

Secondly, surface plasmons can only be optically generated by an evanescent wave whose wave vector matches that of the evanescent SP EM wave, K_{SP} . The latter is given by the following equation:

$$K_{SP} = \omega / c(\epsilon_1 \epsilon_2 / (\epsilon_1 + \epsilon_2))^{1/2} \quad (1)$$

where ω is the frequency of the surface plasmon wave, c is the velocity of light *in vacuo*, and ϵ_1 and ϵ_2 are the complex dielectric constants for the surface active and dielectric emerging media, respectively. The complex dielectric constant, ϵ is directly related to the complex index of refraction $N = c/v = n - ik$ by the following relation:

$$\epsilon = \epsilon' + i\epsilon'' = N^2 \quad (2)$$

where the real part of the complex dielectric constant is $\epsilon' = n^2 - k^2$ and the imaginary part is $\epsilon'' = 2nk$, n is the refractive index, k the extinction coefficient, and v is the velocity of light in the dielectric medium. A component of the incident light vector parallel to the prism/metal interface, K_{ph} , which is described by the following equation:

$$K_{ph} = (\omega / c)\epsilon_0^{1/2} \sin \alpha \quad (3)$$

must be identical with the plasmon wave vector, K_{SP} (equation 1):

$$K_{ph} = K_{SP} \quad (4)$$

The value of K_{ph} can be adjusted to match that of the surface plasmon wave by changing either the frequency (ω) or wavelength (λ) of the excitation light or the incident angle (α). This boundary condition requires that tangential components of the EM fields be continuous across the interface.

These constraints require that plasmons cannot be directly excited by incident light. Furthermore, indirect excitation can only be achieved by an evanescent wave generated by *p*-polarized incident light under total internal reflection. This occurs when the light propagates from a high refractive index incident medium, n_0 (e.g., a glass prism) to a second, emerging medium of lower refractive index (n_2) at incident angles (α) larger than the critical angle, $\alpha_c = \sin^{-1}(n_2/n_0)$. Since the prism has a dielectric constant, $\epsilon_0 = n_0^2$ (i.e., $k_0 = 0$) the following relation must be satisfied: $\epsilon_0 > \epsilon_2$ (or $n_0 > n_2$). Metal-coated diffraction gratings may be used instead of prisms, but require more complex fabrication.

In waveguide techniques the measured interface (or thin film) is placed in the evanescent region of a guided mode propagating in a dielectric waveguide structure. The optical properties of the interface (or thin film) affect this propagation causing changes in the resonant waveguide mode which can be measured by various techniques (attenuated total reflection (ATR) spectroscopy, interferometry, or total internal reflectance fluorescence (TIRF)).

At the resonance condition (equation 4), the incident light is coupled into a SP wave traveling along and bound to the outer active (metal) surface, and the phenomenon is known as surface

plasmon resonance (SPR). The SP wave is nonradiative, and can either decay into photons of the same frequency ω , if coupling by the surface roughness takes place, or be converted to heat.

There are two configurations based on the ATR technique available to optically excite SPs at metal/dielectric interfaces. In the Kretschmann configuration, the prism is in direct contact with the surface active (metal) medium [1]. In the Otto configuration, the prism is separated from the metal by a thin layer (one wavelength) of dielectric [2]. The former technique has wide acceptance due to the ease of thin film fabrication. The latter suffers from the need to precisely control the separation between the prism and metal.

Plasmon-Waveguide Resonance (PWR) Spectroscopy

Similar to SPR, a new technique was developed called plasmon-waveguide resonance spectroscopy [9]. Adding a dielectric (silica) overlayer on top of the silver, surface plasmon and waveguide excitation modes are coupled and linewidths are narrowed. Additionally, both *p*- and *s*-polarized excitations produce resonances allowing direct measurements of anisotropies in the complex refractive index. Refractive index anisotropy reflects changes in mass distribution and therefore changes in molecular orientation and conformation. Absorption coefficient anisotropy reflects changes in orientation of chromophores which may be used in the adsorbed film. See Fig. (1) for a schematic of a PWR device.

One further advantage of the dielectric layer is that it mechanically and chemically shields the metal layer. The relative bandwidths of the two resonances (obtained with the *p*- and *s*-polarized light) and the sensitivity of the measurements can be varied by altering the optical properties of the dielectric layer. The dielectric overcoat can also be used as a matrix where different chemistries can be performed to allow the immobilization of material on the resonator surface.

Theory of Plasmon Spectroscopy

The SPR and PWR phenomena can be understood as a consequence of the application of Maxwell's electromagnetic field theory to thin-film systems [10]. Accordingly, thin-films are characterized by a complex dielectric constant, which includes the refractive index n and the extinction coefficient k , ($n - ik$). In the optical region of the electromagnetic spectrum, this parameter is equal to the ratio of the speed of light *in vacuo* (c) to that in a medium (v), and is numerically equivalent to the optical admittance that is defined by the ratio of the amplitudes of the electric (B) and magnetic (C) fields of the electromagnetic wave [10] according to the equation:

$$Y = C / B = c / v = n - ik \quad (5)$$

where Y is the optical admittance divided by the admittance of free space.

Using Maxwell's equations, the propagation of the plane, monochromatic, linearly polarized, and homogeneous electromagnetic field within a multilayer thin-film system can be described with the following matrix equation [10–12]:

$$\begin{bmatrix} B \\ C \end{bmatrix} = \prod_{r=1}^p \begin{bmatrix} \cos \delta_r & i(\sin \delta_r) / Y_r \\ Y_r / \sin \delta_r & \cos \delta_r \end{bmatrix} \begin{bmatrix} 1 \\ Y_{r+1} \end{bmatrix} \quad (6)$$

where p is the number of layers deposited on the incident medium (in this case the medium for which $r = 0$ is a glass prism, and $(r + 1)$ is an aqueous solution).

$$\delta_r = 2(n - ik)_r t_r (\cos \alpha_r) / \lambda \quad (6a)$$

gives the phase thickness of layer r at the appropriate angle of incidence (α_r) and light wavelength (λ), and

$$y_r = (n - ik)_r / \cos \alpha_r \quad (6b)$$

Equations 6, 6a and 6b allow the examination of the distribution of electromagnetic field amplitudes throughout the thin-film system, as well as calculation of the transmittance, absorbance and reflectance. It also allows the analysis of the resonance phenomena occurring within such thin-multilayer films. The reflectance (R) of such a multilayer system is given by the following relationship involving the admittance:

$$R = (y_0 - Y)^2 / (y_0 + Y)^2 \quad (7)$$

where y_0 is admittance of the incident medium (glass prism). The theory demands that the incident medium should be free from absorption, so that y_0 is real and equals n_0 . Equation 7 describes a reflectance spectrum as a function of the incident angle α of a beam of monochromatic light. Similar spectra can be obtained by varying λ at a constant value of α , or by varying the thickness of the other layers, at constant α and λ . Beyond the critical angle, the emergent wave in the final medium is evanescent and the admittance is imaginary. For s -polarization the admittance is always negative imaginary and so there is normally no corresponding resonance, as is the case in SPR. In PWR, the dielectric overcoat transforms the admittance of the emergent medium to present a positive imaginary value to the metal for both s - and p -polarizations. Depending on the details of the admittance matching overcoat (optical parameters: n , k and t), the system can result in a narrowing or a broadening of the range of angles over which the necessary coincidences are achieved, and hence a similar broadening or narrowing of the resonances. Examination of the distribution of electric field amplitudes through the system shows that the admittance matching layers are important components of the resonator system, similar to cavity layers in narrowband filters or thin-film waveguides in optical couplers.

Properties of the Resonance Spectrum

Figure (2) shows computed angular spectra from Salamon *et al.* [9]. The dashed curve in Fig. (2) panel A, represents the much broader SPR spectrum obtained with the same silver layer as in Fig. (1) but without the dielectric overcoat. This waveguiding layer adds an additional spectroscopic dimension (s -polarized component) and an increased sensitivity, as a consequence of the decreased half-width of both s - and p -polarized resonances. In fact, the resonance linewidth, and therefore the spectral sensitivity, can be adjusted by choosing the appropriate overcoating layers according to the experimental needs, as indicated by the two sets of results shown in Fig. (2B). These spectra result from two different dielectric layer designs as indicated in the figure legend.

Figure (3) shows the electric field distributions obtained with the interface in Fig. (1). The EM fields at the outer interface are 25–50 times larger than the relative incident components. This translates into a high sensitivity for the three parameters describing the resonance spectrum (thickness of the sensing layer, t ; refractive index, n ; and extinction coefficient, k). For a 5 nm thick bilayer the accuracies are better than 1 Å, 0.001 and 0.002, respectively for t , n and k [13]. This accuracy may be limited by the ability to generate a thin sensing film in a reproducible manner. Although there are some alternative techniques for the detection and measurement of small optical changes based on optical waveguides [14], PWR construction is simpler, more convenient and completely isolates the optical probe from the system under investigation.

The resonances can be described by the angle (or wave-length), and the spectral width and depth. These three values depend on three properties of the plasmon-generating and emergent media: the refractive index (n), extinction coefficient (k) and thickness (t). The theory based on Maxwell's equations provides an analytical relationship between the spectral and optical parameters of the film. Despite the tight coupling between refractive index and thickness, one can often find unique values for optical parameters of layers with non-linear least-squares fits [9].

SPR and PWR can be performed in either kinetic or spectroscopic modes. In the simpler kinetic mode, changes in reflected-light intensity are monitored at a single angle as a function of time allowing real-time analysis of binding processes [15]. In the more detailed spectroscopic mode measurement and analysis of entire spectra allows analysis of binding and characterization of structural changes. Steady-state PWR or SPR can determine mass density changes in molecular assemblies accompanying binding, and is often used to determine equilibrium binding constants.

PWR Spectroscopy of Anisotropic Proteo-Lipid Films

Five optical parameters (n and k for p - and s -polarizations and t) can be obtained for each of the three media: plasmon-generating, proteolipid membrane and aqueous buffer solution. For anisotropic films, such as the proteolipid membrane, the thickness (t) represents an average molecular length perpendicular to the plane of the film and will be independent of polarization.

Since common excitation wavelengths are far from the absorption bands of lipids and proteins, nonzero values of k are due only to scattering resulting from coating or proteolipid imperfections.

In contrast, the refractive index will depend on the incident polarization. For uniaxial anisotropic structures in which the optical axis is parallel to the p -polarization direction, the n_p value will always be larger than n_s . This distinction has not always been appreciated in the literature [16]. Furthermore, changes in either n_p or n_s do not necessarily reflect alterations in the absolute mass of the system. They can be partly, or totally, a result of changes in anisotropy. In the case where molecular shape is rod-like (e.g. phospholipids and GPCRs), one can assign two different values to the refractive index: a larger longitudinal one, and a smaller transverse one. In this situation the values of the polarizability, averaged over the whole system and measured either parallel or perpendicular to the direction of the longer molecular axes, will be different. The parallel value will be larger than the perpendicular one. The optical anisotropy (A_n), can be characterized by values of the refractive index measured with two polarizations of light (i.e., parallel, n_p , and perpendicular n_s , to the optical axis) as follows:

$$A_n = (n_p^2 - n_s^2) / (n_{av}^2 + 2) \quad (8)$$

In this equation n_{av} is the average value of the refractive index and, for a uniaxial system is given by:

$$n_{av}^2 = 1 / 3(n_p^2 + 2n_s^2) \quad (9)$$

The anisotropy in the refractive index reflects both the anisotropy in the molecular polarizability and the degree of long-range order of molecules within the system, and therefore can be used as a tool to analyze structural organization (molecular orientation).

From the Lorentz-Lorenz relation, for a pure substance, the mass density d , can be related to n [17,18] by:

$$d = ML = M / A ((n_{av}^2 - 1) / (n_{av}^2 + 2)) \quad (10)$$

where M represents the molecular weight; L the number of moles per volume; and A the molar refractivity of the material. From the thickness and average refractive index value one can calculate the surface mass density, *i.e.*, mass per unit surface area [9]:

$$M = dt = 0.1M / A \{t[(n^2 - 1) / (n^2 + 2)]\} \quad (11)$$

where the thickness is in nanometers and mass in $\mu\text{g}/\text{cm}^2$. Such a simple mass calculation becomes more complicated when the layer is formed from a mixture of substances, as is often the case in real measurements. This can still be dealt with depending upon the specific experimental conditions.

The combination of site-specific chromophore labeling and PWR allows the anisotropies in n and k to be measured in the same sample over a variety of wavelengths. This can provide independent information about the mass and conformational changes occurring in each component of a system. By single-site labeling a GPCR, one could obtain information about conformational changes during binding. Considering GPCRs using this approach, one could independently assign conformational changes occurring in the proteolipid system to the protein or to the lipid and, for example, determine which receptor loop(s) are involved in G-protein binding.

SPR Imaging

In contrast to SPR where either the angle or the wave-length is fixed, and the reflected light is detected by a single-element detector, SPR imaging is performed with both angle and wavelength fixed, measuring the change in reflectance across the sample surface. The reflected light is captured using a CCD camera. Figure (4) illustrates the principle of SPR imaging. This is equivalent to the procedure in any other microscopy which uses plane waves of light or electrons. In the case of SPR microscopy the sample is "illuminated" with surface-bound light rather than with "normal" photons, as is done, for example, in ellipsometric microscopy of thin films. Any Plasmon Surface Polaritons (PSP) and waveguide spectrometer can easily be converted to a microscope by simply adding a lens to the detector side and monitoring the obtained image with a CCD camera. A four-phase Fresnel calculation is used to simulate scanning angle SPR reflectance for different samples at a wavelength of 633 nm [19]. The location of the plasmon resonance shifts to higher angles upon adsorption of molecules. From a pattern sample layer like that sketched in Fig. (4) more light is reflected at an angle of incidence (α_{inc}) from the reactant dots (r_R) and less light is reflected from regions of gold (r_{Au}). An additional shift of the SPR curve occurs upon interaction of analyte molecules with the reactant dot which leads to higher reflectance (r_A). Thus the reactant dots can be monitored across the surface and correlated to their interaction with analyte molecules. The maximal lateral resolution of SPR imaging is determined by the propagation length of the surface plasmon wave and depends on the optical parameters of the prism, the metal layer and the sample. Under optimized conditions a lateral resolution of less than 2 μm could be achieved [20].

Optical effects like light scattering or specularly reflected light affect the contrast of the SPR image. Since s -polarized light is simply reflected by the metal surface, the s -polarized light can be used for a reference image and to enhance the contrast of the SPR image. Only the adsorption of molecules directly on the metal surface or at a short distance of a few nanometers from the surface can be detected because the intensity of a surface plasmon wave decays exponentially normal to the interface of the thin metal layer and the dielectric. For thin dielectric films (thickness < 100 nm) the angle shift is roughly proportional to the changes in refractive

index and thickness of the film. SPR is strongly dependent on temperature (the refractive index of water changes by $10^{-4}/\text{K}^{-1}$). For high sensitive measurements the temperature of the sample must be controlled. The sensitivity and lateral resolution in SPR imaging is affected by the wavelength of the incident light. Sharper resonance curves (narrow width) are observed in the near infrared range than in the red visible wavelength range. A sharper SPR means larger changes in reflectance for changes in refractive index of the sample. The propagation length of the surface plasmon wave is longer in near infrared range than in the red. The use of near infrared light in SPR imaging provides higher sensitivity, but lower lateral resolution. The principle of SPR imaging was first demonstrated by Rothenhäusler and Knoll [21] where SPR imaging was used for the characterization of microstructures within a polymer layer. Recent developments include a SPR imaging transducer for affinity biosensors [22]. The most familiar examples for SPR imaging application are the characterization of adsorption and interaction of biotinylated oligonucleotides, single and double stranded DNA, proteins and polypeptides [23–26]. The size and shape of dots of immobilized biological molecules on a biosensor can be checked by SPR imaging without labeling or staining. Oligonucleotides can be attached either directly or via self-assembled monolayers on the gold surface. If hybridization occurs the refractive index increases, and as a consequence, the intensity of the reflected light is changed. The detection limit of unlabeled RNA and DNA oligonucleotides is as low as 10 nmol/L [27]. SPR images of hybridization and streptavidin adsorption onto spots of different oligonucleotides show that streptavidin/DNA multilayers can be formed and grow by a combination of both DNA hybridization and streptavidin-biotin binding [28]. SPR imaging was used to study DNA arrays and to quantify a high- and low-affinity binding between transcription regulatory proteins and their DNA sequence [29]. Gold deposited on common glass slides are simple and well suitable substrates for this application. Because of the ability of SPR to monitor biomolecular interactions in the near infrared range [30], the characterization of biomolecules on silicon substrates becomes interesting. Recent work describes the application of SPR imaging for screening of ligands in affinity chromatography [31]. The binding and elution behavior of the ligands can be monitored by SPR imaging 15-fold faster and with 130-fold less target protein than conventional chromatographic methods. An interesting example of such a study was performed in which SPR imaging was used to detect the affinity-tagged recombinant proteins expressed in *E. coli* [32]. With regards to model proteins, the hexahistidine-ubiquitin-tagged human growth hormone (His(6)-Ub-hGH), glutathione S-transferase-tagged human interleukin-6 (GST-hIL6), and maltose-binding-protein-tagged human interleukin-6 (MBP-hIL6) expressed in *E. coli* were analyzed. The cell lysates were spotted on gold thin films coated with 11-mercaptoundecanol (MUOH)/dextran, and derivatized with Ni(II)-iminodiacetic acid (IDA-Ni(II)), glutathione, or cyclodextrin. After a brief washing of the gold chip, SPR imaging measurements were carried out in order to detect the bound affinity-tagged fusion proteins. Using this new approach, rapid high-throughput expression analysis of the affinity-tagged proteins was obtained. The SPR imaging-protein chip system was used to measure the expression of affinity-tagged proteins in a high-throughput manner and is expected to be an attractive alternative to traditional laborious and time-consuming methods, such as SDS-polyacrylamide gel electrophoresis (SDS-PAGE), ELISA and Western blots.

One other interesting example of SPR imaging application is for screening affinity ligands. Lowe and coworkers [31] have reported the synthesis, characterization and immobilization of a combinatorial library of 13 ligands to agarose beads and gold SPR devices. Binding and elution behavior were measured against two target proteins, an insulin analogue (MI3) and a recombinant clotting factor (rFVIIa). The binding and elution data from both the SPR and chromatographic systems for both target proteins were comparable with the new SPR screening system.

The kinetics of protein adsorption/desorption onto peptide microarrays has been investigated using real-time SPR imaging [33]. Additionally, the surface enzymatic activities of the protease responsible for the cleavage of a substrate peptide were investigated and the dissociation constants and kinetic data were determined.

All these results demonstrate that SPR imaging is well suited to follow *in situ* adsorption of biomolecules and to quantify adsorption information as well as to study binding events in a miniaturized and high-throughput fashion [34].

SPR-Mass Spectrometry

Mass spectrometry, in particular matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) and electrospray ionization mass spectrometry (ESI), are two of the most powerful tools that are available at present for protein identification. They are used routinely with separation techniques such as 2D gel electrophoresis for profiling-protein expression levels and for target identification. There are still problems with sample extraction and preparation using this approach and, unfortunately, successful identification of a potential drug target does not necessarily lead rapidly to detailed knowledge of protein function. An integral part of proteomics is therefore to examine not only the relative abundance of proteins, but also their potential interactions with each other, that is, to investigate systems biology. Although several technologies exist at the forefront of proteomics, rather predictably, none can single-handedly provide answers to all of the questions. The combination of two or more technologies into one concerted approach is both preferred and essential. In the past decade, the combination of 2D-gel electrophoresis with mass spectrometry (MS) fingerprinting has proven to be the foundation of proteomics laboratories. SPR is used for protein quantification and interaction analysis, and MS is used to delineate the structural features of the proteins. Proteins are affinity captured and quantified from solution via ligands covalently attached to the SPR-sensor surface. Because SPR detection is non-destructive, proteins retrieved on the SPR sensing surface can be further analyzed via MS, either directly from the sensor chip surface, or separately, following elution and micro-recovery. The combination of SPR with MS overcomes the non-discriminatory detection of SPR, enabling identification of: (1) non-specific binding (NSB) of other biomolecules to the surface-immobilized biomolecules (or to the non-derivatized sensor surface itself); (2) bound protein fragments; (3) protein variants (caused by post-translational modifications and point mutations); and (4) proteins complexed with other molecules.

SPR-MS was first demonstrated in 1996 [35] and subsequently described in several proof-of-principle experiments [36,37]. Early attempts at such integration involved the immobilization of a receptor on the biosensor surface that was used to capture the analyte from a complex mixture. The identity of the captured analyte was carried out by 'off-chip' MALDI-TOF [38]. For example, epitope-tagged peptides have been detected and identified directly from *E. coli* lysates at low-femtomole to sub-femtomole levels [39]. Unfortunately, this approach has several limitations: 1) sequence analysis is limited in MALDI-TOF, which is not suitable for analysis of very large or very hydrophobic proteins; 2) the preparation of the sensor chip for MALDI-TOF requires several handling steps; 3) the process destroys the sensor surface, which prevents further processing and analysis; 4) many proteins are post-translationally modified (*e.g.* enzymatic cleavage, phosphorylation, glycosylation) which makes identification on the basis of molecular mass alone much more difficult. To overcome these limitations, a method was developed to elute the captured analyte from the sensor chip *in situ* in the biosensor instrument [40,41]. This approach allows bound proteins to be enzymatically digested *in situ* on the chip and eluted in a small volume of 1% formic acid for direct analysis and identification by ESI-MS/MS and other techniques. Examples of this approach include the isolation and identification of 1,4,5-inositol-trisphosphate-binding-proteins from cell lysates [42],

identification of ligands that bind to orphan G-protein-coupled receptors (GPCRs) using immobilized mammalian-cell membrane fragments [43], the analysis of Staphylococcal toxins in food [44], and the identification of endoproteases using immobilized, affinity-tagged recombinant proteins with unique protease cleavage sites [45].

One of the most intriguing applications of the on-chip SPR-MS approach is in the study of protein complexes. Ligands with affinities towards a protein that is part of an *in vivo* assembled complex can be used as 'hooks' on the SPR sensor to retrieve the entire protein complex from a biological sample prepared under non-denaturing conditions. The SPR sensing offers a unique opportunity to monitor the state of these protein complexes as a function of solvent variations, and subsequent MALDI-TOF MS analysis of the retained components reveals the masses of the constituent proteins, along with any structural modifications present. Recent studies have applied SPR-MS analysis to a protein complex comprising retinol binding-protein (RBP) and transthyretin (TTR) [41].

Another study involved insulin-like growth factors-1 and -2 (IGF-1 and -2) [46], and used SPR-MS to identify the growth factors, either free or in complex, depending on the type of treatment of the human plasma sample. Using antibodies to IGF-1 and -2 immobilized on separate flow cells, SPR-MS revealed the presence of both growth factors (in both flow cells) when the plasma was analyzed under native conditions. By contrast, results obtained during analysis of the denatured plasma revealed the presence of each target growth factor in its corresponding flow cell only. These results strongly suggest the presence of an *in vivo* complex containing both IGF-1 and IGF-2. In addition, a novel, truncated IGF-2 variant missing its N-terminal alanine was also identified in the analysis.

In summary, given the dual aspect of the analysis (quantitative and qualitative), SPR-MS holds great promise for the investigation of protein complexes, and, when implemented rigorously, might yield insight into the mechanisms of protein complex assembly and the function of each specific protein.

IMMOBLIZATION TECHNIQUES OF THE ANALYTE UNDER STUDY

The interface between the sensor surface and the chemical or biological system to be studied is a key component of optical biosensors. Proteins need to be attached to some form of solid support, while retaining their native conformation and binding activity. The attachment must be strong enough so it is stable over the course of a binding assay, as well as providing sufficient and uniformly reactive binding sites to the solution phase for interaction with the analyte. Most crucially, the support should be resistant to non-specific binding of the sample, which can mask the specific binding signal.

Many coupling strategies use a chemical-linker layer between the sensor base (*e.g.*, gold layer) and the biological molecule of interest. Self-assembled monolayers composed of functionalized alkane thiols [47] and alkoxy silanes [48] have been formed on planar surfaces acting as ideal linkers. The alkyl termini of these molecules can be derivatized with ethyleneglycol subunits to produce a planar surface that is resistant to non-specific binding of proteins [49], or can be mixed with molecules that have suitable chemical reactivity for protein capture (examples: epoxy, carboxyl, amino, biotinyl, nitrilotriacetic acid groups [49–51]). Usually, the larger binding partner is immobilized on the surface, and the smaller binding partner is allowed to bind to the surface from free solution, in order to reduce mass transport effects (this will be further discussed below) but the opposite configuration has been performed. The chemical-linker layer can be used as a substrate for the attachment of a polymer coat or hydrogel that renders the surface highly resistant to non-specific absorption of molecules. The same polymer also provides a 3-D scaffold for protein immobilization. The most widely used

biosensor polymer coat is carboxymethyl dextran [52] which is covalently attached ($1\text{--}3\text{ ng/mm}^2$) to form a flexible hydrogel of estimated thickness $100\text{--}200\text{ nm}$ [53] or $200\text{--}400\text{ nm}$ [54]. As described below, there are several strategies for either covalent or non-covalent attachment of molecules to either the planar self-assembled surfaces or polymer coats. The choice of the coupling chemistry requires a careful consideration of several aspects: the resultant orientation of the molecules, its local environment on the surface, the stability of the linkage under the conditions used to regenerate the surface, and possible effects of the coupling chemistry on components of the binding interaction. A special section in this manuscript will be dedicated to the preparation of surfaces containing membrane proteins since they represent a major subject in this review and pose uniquely challenging properties.

Coupling Methods for Protein Immobilization: Covalent Attachment

The dextran matrix described can be derivatized with a number of different functional groups and this allows for a variety of immobilization chemistries [55–59] (see below). A regeneration procedure for this sensor chip has been described [60]. The particular advantage of using the flexible, hydrophilic dextran matrix is that it provides better accessibility between the immobilized macromolecules and their binding partners and potentially increases the SPR sensor signal, by virtue of a relatively large number of immobilized binding sites and more efficient use of the evanescent field [52,61]. A widely applied immobilization procedure utilizes activation of the carboxy groups of the dextran gel with a mixture of N-hydroxysuccinimide (NHS) and N-ethyl-N'-(dimethylaminopropyl)-carbodiimide to form NHS esters, which enables coupling to the amino groups on proteins [62]. The effectiveness of the coupling relies on a pre-interaction of the protein by electrostatic attraction to the negatively charged matrix at pH values below the pI of the protein [52,62]. Acidic receptors (with an isoelectric point (pI) <3.5) are difficult to immobilize by amine coupling, as the low pH required for electrostatic pre-concentration to the sensor surface protonates the primary amino groups and reduces the coupling efficiency. The use of low pH also increases the possibility of protein denaturation [59]. Another disadvantage of the NHS-ester immobilization procedure is the potential occurrence of random coupling to different lysines on the protein [58], leading to a heterogeneous population of proteins with random orientation on the surface, exposing diverse binding sites with different accessibilities and reactivities. A more homogeneous population of oriented proteins on the surface can be prepared by a variety of more specific immobilization methods, such as coupling by thiol/disulfide exchange [55,57], aldehyde coupling [57], hydrazine coupling [58], and sulfhydryl group coupling [58]. In several studies, for example, a specific orientation of the immobilized receptor domain was achieved by creating a thiol coupling/disulfide bond to cysteine residues that were introduced into one reactant via site-directed mutagenesis [55,63,64]. For immobilization of proteins with hydrophobic anchors, modification of the dextran matrix by heptyl residues has been described [59]. Covalent linkage could be achieved through a perfluorophenylazide-derived hydrophobic crosslinker [59].

Coupling Methods for Protein Immobilization: Non-Covalent Attachment

Metal coordinating groups such as iminodiacetic acid (IDA) and nitrilotriacetic acid (NTA) have been widely used for the direct immobilization of $6 \times \text{His-}$ and $10 \times \text{His-}$ tagged proteins [47,65,66]. In brief, the procedure consists of covalently binding the chelator IDA or NTA to the sensor surface and subsequently loading it with divalent cations (*e.g.*, Zn^{2+} , Cu^{2+} , Ni^{2+}). The free coordination sites of the chelator-metal complex are then filled by additional electron donating groups such as histidine residues in the sequence of a natural or recombinant protein (see Fig. 5). The formation of the protein-NTA chelator complex is highly specific with reasonable affinities, and furthermore, it is fully reversible upon addition of a competitive ligand (histidine, imidazole), by protonation of the histidines, or by removal of the metal ion by EDTA complexation.

The method has been successfully applied on gold surfaces for SPR detection in the case of water-soluble proteins such as enzymes [50] and Fab fragments [56]. A similar approach was used for the binding of histidine peptides and proteins to chelator-lipid monolayers at the air-water interface [67,68]. The method has also been applied to membrane proteins. The serotonin-gated ion channel 5-HT₃ was immobilized in a detergent-solubilized form on quartz glass surfaces and real-time ligand-binding monitored [66]. The use of the this affinity-tag has a great potential as the His-tagged receptor of interest can be bound to the sensor surface by just incubating the sensor surface with raw detergent extracts of cells. Since SP techniques are highly sensitive, the extract of a few cells expressing the protein of interest will be sufficient for those assays [69]. The histidine-tag approach allows a controlled, unidirectional immobilization of membrane proteins in a lipid bilayer, either directly by fusion of lipid vesicles containing the receptor, or, alternatively, by formation of a supported lipid bilayer after detergent-solubilized receptor has been immobilized.

Indirect coupling, exploiting the high affinity of the avidin-biotin interaction, can be achieved by immobilizing avidin/streptavidin to the dextran matrix and binding biotinylated macromolecules [57,58,70,71]. This method has the additional advantage of not requiring pre-concentration in the matrix, being highly efficient and producing stable complexes, but it has the disadvantage of being effectively irreversible. It has been preferred for the immobilization of DNA and RNA [71–74] and also to immobilize lipid vesicles [74].

Monoclonal antibodies can be covalently attached to a solid support by means of amine coupling, as described above. Epitope-tagged or fusion proteins can be directly and reversibly coupled to the surface through antibody-antigen interaction [75–77]. Commonly used tags include, for example, glutathione S-transferase, *herpes simplex* virus glyco-protein D epitope, FLAG epitope and the 6x His, previously discussed.

For SPR experiments performed using the commercially available Biacore instrument there is a variety robust and reproducible surfaces available [78]. In Table (1) is a list of the surfaces currently available from Biacore and Affinity Sensors along with their general application.

FUNCTIONAL RECONSTITUTION OF MEMBRANE-BOUND RECEPTORS

Many of the interactions that are probed in the drug discovery process occur with membrane-bound receptors, such as ligand-gated ion channels, GPCRs, antibody receptors and cytokine receptors. Almost 50% of the drugs on the market target G-protein coupled receptors (GPCRs). To better understand the binding mechanisms of ligands with these receptors, the ligand-receptor interactions must be probed directly *in vivo* or in reconstituted membrane systems [43,69,79]. Most techniques for detailed kinetic analysis of molecular recognition events are applied in solution phase using a truncated, soluble form of the receptor. Membrane receptors, however, have significant hydrophobic domains, and can have different tertiary structures and binding affinities in solution relative to those that occur in a membrane environment. This type of approach is therefore limited to receptors that contain a single transmembrane domain. Further, it does not allow the study of ligand induced signal cascades, or the investigation of complex membrane proteins, which often homo- or heterodimerize. In response to these challenges, there has been substantial progress over the past ten years in the development of techniques that allow the analysis of membrane associated ligand-receptor interactions in a model that resembles their native membrane environment. Biophysical techniques, such as patch clamping, magic-angle-spinning NMR, fluorescence-correlation spectroscopy, fluorescence resonance energy transfer and analytical ultracentrifugation, have been applied to the analysis of binding to whole cells, membrane protoplasts and proteoliposomes. However, interactions with membrane receptors are surface-related processes that are difficult to study with bulk techniques and furthermore, some of those techniques require large amounts of

protein which cannot yet be easily obtained. Furthermore, there are relatively few methods that allow quantitative, non-invasive determination of both the affinity and the kinetics of such interactions. Many researchers have immobilized membranes on a sensor surface to achieve this goal. Below, different strategies are presented for the immobilization of membranes on sensor surfaces.

Supported Lipid Monolayers

The simplest method for the immobilization of membranes on a sensor surface is the adsorption of lipid onto a hydrophobic surface [80]. This results in the formation of a supported lipid monolayer, with the hydrophobic acyl chains of the lipids contacting the hydrophobic surface, and the polar lipid head groups facing the solution. For the formation of such hybrid bilayer membranes, the sensor surface has to be properly modified. It may consist of a physisorbed monolayer of fatty acid salts or a chemisorbed monolayer of alkyl chains linked to the support via functional groups, or a hydrophobic hairy-rod polymer [81]. Alkylthiols on gold are excellent substrates; dense monolayers of lipids or other amphiphiles are readily formed by dipping the hydrophobized sample vertically or horizontally through a phospholipid monolayer at the air-water interface of a Langmuir trough which allows control of the lipid packing density. This surface density influences lipid order in supported Langmuir-Blodgett monolayers, lateral diffusion and the efficiency of recognition reactions. Supported monolayers in the fluid state on alkylated surfaces have lateral diffusion coefficients that compare well with those seen in lipid bilayer leaflets [82].

This method is generally limited to receptors (peripheral and partially inserted) that are anchored only in the outer leaflet of a native membrane, or to cases in which the analyte binds to the lipid itself. The hybrid bilayer membranes comprising of a surface-fixed self-assembled monolayer and a second fluid lipid monolayer, are useful for studying bio-molecular interactions on the membrane surface. They are, however, not suitable for the insertion of transmembrane proteins in a functionally active form. This is due to the particular structure of these layers, which are too rigid, containing an unfavorable high proportion of hydrophobic to polar regions, and most importantly, being totally attached to the solid support thus excluding the space and water required for the proper structure of the outer membrane parts of membrane proteins.

The use of optical biosensors with supported lipid monolayers was first exploited by Vogel and co-workers to study the interaction of the *cholera toxin* B subunit with the cell-surface ganglioside GM1 [80] a receptor-ligand pair that has been used widely to validate model membrane systems [83,84].

Tethered Lipid Bilayers

As mentioned in the previous section, a lipid layer that is directly adsorbed onto a surface has the significant drawback of not being able to accommodate transmembrane proteins with sizable cytosolic or extracellular domains. To overcome this limitation, several methodologies have been developed to space a lipid bilayer at some distance away from the surface. Membranes can be covalently attached to solid supports, either directly by suitable reactive lipid polar head-groups, or via amphiphilic copolymers. Vogel and coworkers [85] reported the synthesis and use of a thiolipid comprising a hydrophilic oligoethoxy spacer between the lipid headgroup and the anchoring sulphur groups to form tethered lipid membranes on gold surfaces. Starting from thiolipid monolayers, bilayers were formed by self-assembling phospholipids using lipid vesicles or mixed micelle dilution procedures. These 'tethered bilayer membranes' (tBLMs) have a number of advantages over commonly used simple supported bilayers. Since the lipids are covalently fixed they make an extremely robust bilayer lasting for long periods of time. The spacer acts as an elastic buffer, decoupling the lipid layer from

the solid support. The hydrophilic nature of the spacer ensures the provision of a water film between the lipid layer and the solid support, which is essential for retaining the functional activity of membrane-traversing GPCRs inserted into these bilayers.

Those tethered bilayers can be probed not only by SPR, surface plasmon fluorescence spectroscopy (SPFS), resonant-mirror and waveguide techniques [86], but also can be analyzed with electrical measurements if the surface is conducting (for example, metals, indium-tin oxide and conducting polymers).

Vogel and co-workers have used a thiophospholipid that has a triethyleneglycol spacer unit to capture membranes and membrane proteins [85]. This approach has enabled the functional reconstitution of membrane proteins such as rhodopsin [51,69] in laterally patterned tethered lipid membranes where receptor activity and transducin interaction were monitored. This section is further discussed in the section dedicated to SPR applications.

A similar approach was used by Naumann *et al.* [87], who introduced thiolipids with a peptide spacer (instead of ethoxy chains) to prepare dilute anchoring layers for bilayer deposition and incorporation of F_0F_1 -ATPases. Cornell and co-workers, with the addition of a membrane-spanning thiophospholipid that greatly improved the stability of the tBLM, have investigated ion-channels conductance [88]. The *Torpedo californica* acetylcholine receptor was incorporated into a thiopeptide supported lipid bilayer by fusion of receptor proteoliposomes with a gold-supported thiopeptide lipid monolayer. The fusion process and specific binding of the antagonist was monitored by SPR [89].

Williams and co-workers achieved anchoring of bilayers by spreading phospholipid vesicles on gold surfaces carrying immobilized cholesterol derivatives in different densities [90]. A modification of this technique is bilayer formation and anchoring in one step. It consists of incubating the surface with mixed lipid vesicles containing a certain fraction of lipids with surface-active headgroups. This had been reported for mixed thiolipid/phospholipid vesicles on bare gold [91], and mixed vesicles from succinimidyl-conjugated dialkylamine and phospholipid on cysteinamine-covered gold [92]. A new variation of this system is the use of His-tagged lipids as anchor lipids which can be reversibly coupled to metal-chelating surfaces [93].

Solid-Supported Bilayer

A Teflon spacer has been used by Tollin and co-workers to support bilayers over a prism surface, of a SPR/PWR sensor, where GPCRs are inserted and signaling is investigated [9]. The principles behind the generation of a self-assembled solid-supported lipid membrane are the same that govern the formation of the so-called black lipid membrane prepared by Montal-Mueller-Rudin procedure [94]. Briefly, a lipid bilayer forming solution is spread across a Teflon orifice which separates the silica waveguide surface from the aqueous phase in the PWR cell [9]. The hydrophilic SiO_2 surface is covered with a thin layer of water of condensation [48,95] and attracts the polar groups of the lipid molecules with the hydrocarbon chains oriented toward the bulk lipid phase, which induces an initial orientation of the lipid molecules. The next step involves addition of aqueous buffer into the sample compartment of the PWR cell, which results in formation of a plateau-Gibbs border that anchors the membrane to the Teflon spacer. This border allows membrane flexibility wherein the lipid molecules can get displaced or recruited upon insertion of a membrane protein or as a result of ligand-induced protein conformational changes. GPCRs such as rhodopsin [96–98], the human delta opioid receptor (hDOR) [99–102] and the β -adrenergic receptor [103] have been incorporated into this bilayer while keeping their activity, and allowing conformational changes of the protein upon ligand or other protein interaction to be monitored by SPR and/or PWR in real time in a favorable environment for the functionality of those proteins. The interactions of phosphatidylserine (PS)

synthase [104] and penetratin [105] with lipid membranes have also been monitored by this methodology. The studies with GPCRs will be further discussed in the section dedicated to SPR/PWR applications, as well as the advantages and disadvantages of the use of this lipid bilayer system.

Polymer-Supported Bilayers

To overcome problems associated with roughness of the underlying surface, lipid bilayers can be bound to, but structurally decoupled from, the solid support, by first coating it with a flexible polymer. These soft polymer cushions provide a hydrated lubricating layer between the surface and the membrane that allows the 'self-annealing' of surface defects. In this manner, the polymer cushion resembles the cytoskeleton that anchors the plasma membrane of a cell. There has been a great deal of work investigating the polymer-bilayer interface that resulted in the formation of softly supported bilayers [106–107]. These layers are held to the water-swollen polymeric support by weak adhesions and should provide a native like environment for active membrane proteins. In order to increase the stability of such complex systems, one may covalently link the polymer support to the substrate. Additional strength can be gained by reacting alkyl chains to the soft support. Three basic polymer-based strategies have been used: chemical grafting to a surface of carboxymethyl dextran that is modified with alkyl chains, which are needed for anchoring to lipids and membranes [108]; coupling of lipopolymers that have functionalized hydrophobic head groups to a surface [71]; or the use of copolymers that have reactive disulphide groups to anchor the polymer to a gold surface and present amphiphilic groups with long alkyl chains to anchor membrane fragments and vesicles [109–110]. These model membrane surfaces can be used for extremely rapid (for example, 1 min) functional reconstitution of membrane fragments and detergent-solubilized GPCRs 'on a chip' [43,111].

Microarrayed Lipid Layers

With the numerous orphan GPCRs and other novel membrane-receptor targets that now have been cloned and overexpressed, there is considerable interest in methods to create spatially addressable membrane arrays. Much of the pioneering work in this area has been carried out by the groups of Boxer [112] and Cremer [113]. Both groups used a process called microcontact printing (μ CP), in which a patterned stamp that is made from poly(dimethylsiloxane) (PDMS), is brought into contact with a planar supported lipid bilayer. This results in the displacement of adsorbed lipid at the areas of contact between the stamp and the slide, which can then be filled with a blocking molecule such as bovine serum albumin (BSA). In an alternative approach, Boden and co-workers [114] have formed mixed, self-assembled monolayers of hydroxyl- and cholesterol terminating thiols to capture lipid bilayers, which can be microarrayed using μ CP techniques.

POINTS TO CONSIDER WITH REGARD TO EXPERIMENTAL PROCEDURES AND DATA ANALYSIS WHEN USING SP TECHNIQUES

Possible Effects of the Immobilization

Obviously, the immobilization of reactants to the sensor surface can, in some cases, interfere with their binding properties, for example, by inducing conformational changes in the binding sites or sterically restricting the access of the binding partner. If nonspecific immobilization chemistry such as amine coupling is employed, multiple subpopulations of different orientations and different affinities could be produced [115]. This may result in a broadening of the binding isotherm in the equilibrium data, where the binding kinetics could be mistakenly characterized by multi-exponential binding-progress curves; each exponential term reflecting binding to a subpopulation of independent binding sites [116]. On the other hand, multi-exponential binding can also be a result of mass transport limitations and non-uniform

distribution of the binding sites within the immobilization matrix [115]. Therefore, this ambiguity should be resolved experimentally, by means of control experiments for mass transport limitations (see below) and for artifacts of immobilization. A different choice in the employed coupling chemistry, solution competition experiments, and the complete characterization of the binding isotherm by equilibrium experiments constitute potentially useful tools. Another possible effect of the immobilization, which is related to the high surface density of binding sites in biosensor experiments, is that some immobilized macromolecules may have the potential for oligomerization [63]. If binding properties change with the oligomeric state or if the mobile reactant is multivalent [63], the results of a biosensor experiment may depend on the surface density of the immobilized species.

Steric Hindrance

It has been noted that high local concentrations of immobilized reactant at the sensor surface can lead to steric hindrance in binding to neighboring binding sites [15,115,116]. In several studies, high macromolecular concentrations of up to 100 mg/mL and more within the immobilization matrix [15] would make steric hindrance and excluded-volume effects more than likely. In some respects, this would affect the binding process in a way similar to that of subclasses of differently accessible binding sites. Both lead to a broadening of the binding isotherm and have the smallest impact on the binding kinetics at low saturation of the binding sites. However, the measured dissociation rate can be expected to be less affected for the excluded-volume effect. Edwards and coworkers [116] compared the binding kinetics of human serum albumin to an antibody immobilized either in a dextran matrix or on an aminosilane surface. In the presence of the immobilization matrix, the data were well described by a double exponential, while the binding to the aminosilane-bound binding sites was described by a single exponential. Concluding that steric hindrance affected binding within the dextran matrix, the investigators interpreted the fast component of the binding progress as representative of binding events similar to those in solution; they interpreted the slow component as produced by artifacts of steric hindrance or restricted access to the binding sites. A change in the density of immobilized binding sites can diagnose and eliminate the effects of steric hindrance. It should be noted that the direct attachment to a planar surface in the absence of an immobilization matrix alone does not necessarily eliminate these problems, since the effects of excluded area might persist at high surface densities.

Mass Transport and Bulk Effects

Since optical biosensors generally require a surface-immobilized receptor to function, it therefore is important to consider the possibility of mass-transport-limited binding between the analyte in bulk solution and the receptor on the sensor surface. Such binding can occur when the binding of the analyte to the receptor is faster than the diffusion of the analyte from the bulk solution to the receptor at the surface. The same phenomenon also results in increased rebinding of the analyte in the dissociation phase, as the released analyte can rebind to free receptor before it diffuses into the bulk solution. The effect is most pronounced with very large analytes (which have low diffusion rates), and with those that have very fast association rates (comparable to the diffusion rate). To minimize the effect, very low levels of receptor are immobilized and high flow rates are used, which reduce the depth of the surface-associated 'unstirred' boundary layer [75]. It is also possible to introduce a 'mass transport' rate constant into fitting algorithms to ensure that the binding data is correctly analyzed [117]. Results using a computer simulation have led to the suggestion that the carboxymethyl-dextran hydrogel, which is most commonly used with optical biosensors, could significantly retard the diffusion of analyte to its receptor at the surface [15]. This supposition has subsequently been shown to be incorrect, as identical rate constants were obtained for analyte binding when a receptor was immobilized on either a carboxymethyl-dextran hydrogel or a planar, self-assembled monolayer [75]. When using an optical biosensor, it is extremely important to include blank

surface controls and, if possible, non-relevant receptor controls, to correct for the effects of signal drift, non-specific binding and other bulk effects. The carboxymethyl-dextran matrix that is normally used for screening small molecules is a negatively charged hydrogel. In water and buffers, the carboxymethyl-dextran chains repel each other, which lead to expansion of the hydrogel and a change in mass distribution in the evanescent field near the surface. In addition, when slightly different amounts of a receptor are immobilized on different surfaces or spots, there are subtle differences in the amount of repulsion, and the hydrogel can shrink or swell accordingly [75]. This does not normally affect assays that are carried out in biological buffers, as bulk refractive changes can be corrected successfully by subtraction of a reference surface that contains no receptor, or a non-relevant control receptor. However, small compounds are often prepared and stored as 1–10% dimethylsulphoxide (DMSO) solutions. When using DMSO solutions, the bulk effects that arise from the variation in hydrogel void volume (that is, the space that is not occupied by receptor) can mask the specific binding signal. It is possible to circumvent this problem by first creating a calibration curve using varying concentrations of DMSO in running buffer in the absence of the small molecule. This 'normalizes' for the bulk refractive index changes on the different surfaces, and high-quality binding data can then be obtained [118].

APPLICATION OF SURFACE PLASMON TECHNIQUES FOR THE STUDY OF SIGNAL TRANSDUCTION SPR

Rhodopsin is the GPCR that has been most widely used in Plasmon Resonance studies and so an extended section will be devoted to this protein. There are two reasons for the large number of studies performed with this receptor. Firstly, the visual signal cascade is biochemically and biophysically well-characterized, and secondly the rhodopsin and other components of the cascade are available in high purities and large amounts. Although triggered by light, the visual cascade shares many properties with the signal cascades of ligand-activated GPCRs, for example, the mechanism of receptor conformational changes following the trigger, the role of the cytoplasmic surface of receptors in G-protein coupling, and receptor shut-off mechanisms. Rhodopsin itself has been studied using a variety of biophysical techniques, giving information on its structure in the membrane [119], on its rotational and translational diffusion [120–122], and on the influence of lipid environment on its function [123–125]. The activation of rhodopsin by light offers a unique opportunity to resolve fast reactions in the signal amplification cascade without the need to consider ligand accessibility and orientational effects. Knowledge of the major partial reactions and the relative simplicity and linearity of the signal pathway make an interpretation possible in terms of quantitative models.

Rhodopsin signal transduction studies using SPR were first investigated by Tollin and co-workers about ten years ago using procedures and methods generally applicable to any GPCR [96,97]. In these studies, the purified, and octylglucoside-solubilized receptor was incorporated into a solid-supported lipid bilayer, made across a small hole in a Teflon block, by detergent dilution below the critical micelle concentration (cmc) (more detailed information on the preparation of such lipid membranes has been provided above). The magnitude of the SPR changes caused by light was found to vary with pH in a manner parallel to flash photolysis experiments, and thus monitors the formation of the photoactivated intermediate metarhodopsin II (MII). Fitting experimental spectra to theoretical ones has provided information about the refractive index and thickness of bilayer occurring before and after light activation. The activation of rhodopsin was found to produce a 4–6 Å increase in thickness consistent with an elongation of rhodopsin and exposure of recognition sites for G-protein [96,97]. The average surface area occupied per rhodopsin molecule was also calculated in further detailed studies from the surface concentration of lipid-incorporated protein [97]. An average surface area per molecule of 1260 Å² was obtained, in good agreement with rhodopsin

dimensions determined by other techniques such as electron cryomicroscopy [119] and X-ray crystallography [126,127]. Transducin binding to rhodopsin in the dark-adapted state was monitored showing that transducin binds tightly ($K_{eq} = 60$ nM) and in a positively cooperative manner to form a closely packed film [97]. This work also showed that transducin binding saturates at a transducin/rhodopsin ratio of approximately 0.6. Additionally, spectral changes were observed upon GTP addition to the proteolipid film, after transducin binding, which were correlated with the dissociation of the α -subunit of Gt (transducin), formation of a new MII-Gt complex, and possible conformational changes of Gt as a consequence of complex formation.

The use of solid-supported lipid bilayers constitutes an ideal system for SP investigation of GPCRs signal transduction for the following reasons: 1) it provides a very fluid environment where lipids can move in and out of the lipid bilayer as needed due to the natural existence of the Plateau-Gibbs border; 2) the lipid membranes are easily prepared and stable and can be prepared from a variety of lipid compositions, expanding the use of this technique to lipid-protein interaction studies; 3) the proteins to be incorporated into the bilayer do not need to be chemically modified as they are not attached to the plasmon surface; 4) since the bilayer is not covalently attached to the surface of the sensor it can be easily cleaned with detergent and reused many times; 5) the protein is incorporated into the lipid bilayer in a uniform manner, that is with the longer axis perpendicular and shorter axis parallel to the membrane plane. The molecules can be inserted into the lipid bilayer with either the N-terminus or the C-terminus facing the aqueous side of the membrane [101]. Whereas this last point may be interpreted by some as disadvantageous as a non-homogeneous population of receptors is created, it can actually be advantageous as it allows one to bind either/both ligand and G-proteins (or other molecules that bind to GPCRs through the C-terminus face) to the proteolipid system expanding the range of studies that are possible with this technique. One disadvantage of this setup is that since there is a very small space between the lipid bilayer surface, mainly due to the presence of water molecules and irregularities in the silica surface, part of the membrane proteins incorporated may denature and their lateral mobility may be decreased.

A different strategy for the immobilization of GPCRs on solid supports has been developed by Vogel and co-workers [16] and applied to studies with rhodopsin. They have prepared anchored lipid membranes in a novel way to create micropatterned arrays of fluid lipid bilayers. They have shown that these bilayers are suitable for the functional reconstitution of the membrane protein rhodopsin. The concept is depicted in Figure (6). The lipid membrane on the gold surface of the SPR transducer consists of micrometer-sized regions of fluid phospholipid bilayers separated by fluid phospholipid monolayers (assembled on a rigid, gold-attached thiolipid monolayer). The fluid lipid bilayer thus serves as a suitable matrix for reconstituting the transmembrane receptor protein in a functionally active form, whereas the fluid lipid monolayer regions should hinder the insertion of receptors. The G-protein, in this case transducin, simply partitions to the membrane water interface and is uniformly distributed over the entire outer surface of the supported lipid mono and bilayers. In this work they have reported that rhodopsin was found to incorporate preferentially into the phospholipid bilayer regions, whereas transducin was uniformly distributed over the entire outer surface of the supported patterned membrane. Coupling reactions with transducin were found to closely resemble those of the native system, including the disassembly of the G-protein subunits upon GTP interaction, indicating that the native functionality of rhodopsin was restored in the supported membranes. Activation of rhodopsin by light and nucleotide exchange (GDP-GTP) on the transducin α -subunit leads to dissociation of the α -subunit from the $\beta\gamma$ dimer and their release from the membrane. Consequently, transducin is depleted in bilayer regions, but not in monolayer regions, which results in a lateral gradient of membrane-bound transducin. Transducin diffuses along this gradient from monolayer to bilayer domains. Such lateral displacement of protein mass and its release from defined locations on the membrane can be

observed by one-dimensional imaging SPR. An influence of rhodopsin on the dark binding of transducin to lipid membranes could be demonstrated. This method is suitable for the investigation of lateral diffusion of transducin, and can be extended to include signaling proteins downstream of the G-protein, and may be applied to functional screening of other G-protein-coupled receptors.

Following the idea of micro-patterned bilayers, Vogel and co-workers have immobilized rhodopsin in a patterned, oriented and functional manner that facilitates surface-sensitive detection. These patterns include receptor-free areas to be used as reference ("local referencing"), enhancing the sensitivity of the assays. The glycosylation site of rhodopsin close to the extracellular N-terminus was exploited by using carbohydrate-specific chemistry [65] for biotinylation, thereby confining the biotin tag to the extracellular domain of the receptor (Fig. 7). This resulted in uniform orientation upon immobilization, where the intracellular domain of the receptor pointed away from the surface, optimizing the interaction with the G-protein. The sensor chip was covered with a mixed self-assembled monolayer (SAM) consisting of biotinylated thiols. An excess of hydroxy-undecanethiol (HTA), to which streptavidin was bound. Micropatterns of the receptor were created using microcontact printing [128] to pattern the SAM with respect to the biotinylated thiols, resulting in micropatterns of streptavidin. After immobilization of the receptor and washing with detergent, a supported lipid bilayer (SLB) [79] was formed by micellar dilution [85], followed by addition of the G-protein. The activity of the receptor was detected based in the SPR signal, observed upon addition of GTP to the proteolipid film consistent with the dissociation of the G-protein followed by a reassociation signal with G-protein relaxation to its resting state by hydrolysis of the bound GTP. In these studies the capability of rhodopsin to bind its ligand, retinal, was also investigated. To test for ligand binding the immobilized rhodopsin was converted to opsin by repeated flashes. Then, either 11-cis- or 9-cis-retinal was added and allowed to bind to the receptor. The desorption signal upon photoactivation revealed that 60% or 90% of the initial activity was regained. Based on this result, they hypothesized that an excess of chromophore over receptor should lead to a system in which the activity is constant after each flash, because all generated opsin is reconverted to rhodopsin. Indeed, the same rhodopsin activity was measured during long periods of time (up to 6h) and during several activation cycles at sufficient concentrations of 11-cis- or 9-cis-retinal (10 mM). The results demonstrate the high activity and stability of the reconstituted receptor G-protein in this system.

A straightforward method for the rapid immobilization and reconstitution of GPCRs on carboxylated dextran surfaces modified with long alkyl groups has been presented recently by Karlsson and Löfås [111]. The detergent-solubilized receptor was coupled by the amine group upon injection of lipid/detergent-protein micelles over the immobilized surface. The detergent is rapidly removed by flowing detergent-free running buffer over the sensor surface. This work demonstrated that there is an optimal detergent to lipid ratio for the optimal protein capture. This method is simple and rapid (protein is reconstituted in the surface in ~ 1 min) being optimal for applications demanding standard and high throughput processing such as screening and array assays. Although, it suffers from the disadvantage of the formation of a non-uniform population of receptor molecules (amine attachment may occur through different amines in the protein).

The dimmer species (Mr 580 000) of the nicotinic acetyl-choline receptor, isolated from the electric organ of *Torpedo californica*, was incorporated into a thiopeptide supported lipid bilayer by fusion of liposomes with reconstituted receptor onto a gold-supported thiopeptide lipid monolayer [109]. Surface plasmon resonance spectroscopy was used to monitor in real time the fusion process as well as the specific binding of the antagonist α -bungarotoxin. Surface plasmon fluorescence spectroscopy [129] was then used to monitor subsequent binding of the

monoclonal and fluorescent labeled polyclonal antibodies. The different binding assays indicate successful incorporation of receptor in the lipid bilayer.

In a recent work, the chemokine receptors CXR4 and CCR5, with a C-terminal C9 tag, were captured on a carboxymethylated dextran chip modified with hydrophobic alkane groups and the monoclonal antibody ID4 (binds specifically C9 tag) [130]. The capture occurred from detergent-solubilized receptors in crude cell preparations onto the bio-sensor surface followed by reconstitution in a lipid environment. The conformational states of the receptor was confirmed using conformational dependent antibodies, and by characterizing the binding properties of a native chemokine ligand. The method has the great advantages of not relying on a pure sample preparation and of leading to a homogeneous receptor preparation.

PWR

PWR was recently applied to probe the effects of lipid composition on conformational changes of rhodopsin induced by light and due to binding and activation of transducin (G_t) [98]. In those experiments octylglucoside-solubilized rhodopsin was incorporated by detergent dilution into solid-supported bilayers, composed either of egg phosphatidylcholine (PC) or various mixtures of a nonlamellar-forming lipid (dioleoylphosphatidylethanolamine; DOPE) together with a lamellar-forming lipid (dioleoylphosphatidylcholine; DOPC). Light-induced proteolipid conformational changes as a function of pH were measured and the pK_a 's for the MI-MII equilibrium were determined. Results obtained correlated well with previous flash photolysis studies, indicating that the PWR spectral shifts monitored metarhodopsin II formation [125]. The magnitude of these effects, and hence the extent of the conformational transition, was found to be proportional to the DOPE content. These results are consistent with previous suggestions that lipids having a negative spontaneous curvature favor elongation of rhodopsin during the activation process [125]. In addition, measurements of the G_t /rhodopsin interaction in a DOPC/DOPE (25:75) bilayer at pH 5 demonstrated that light activation increased the affinity for G_t from 64 nM to 0.7 nM, whereas G_t affinity for dark-adapted rhodopsin was unchanged (see Table (2)). By contrast, in DOPC bilayers the affinity of G_t for light-activated rhodopsin was only 18nM at pH 5. Moreover exchange of GDP for GTP γ S was also monitored by PWR spectroscopy. These studies demonstrate the ability of PWR spectroscopy to monitor both mass density and conformational changes related to the lipid modulation of GPCR activation and signal transduction events. This will be especially important in applications to GPCRs for which flash photolysis techniques cannot be used. In addition, the observation that the lipid composition of the bilayer can enhance the ability of a GPCR to become activated and to bind a G-protein has important implications for the regulation of receptor activity by sorting into membrane micro-domains [131].

Several aspects of the signal transduction pathways for the human delta opioid (hDOR) receptor have been investigated by our laboratories using PWR. In those studies the purified and detergent-solubilized receptor has been incorporated into a solid-supported lipid bilayer that is mounted across a small hole in a Teflon block which is in direct contact with the prism surface. The receptor has been shown to be active after incorporation into the lipid bilayer, in the PWR cell, based in its ability to recognize different classes of ligands with affinities very similar to the ones presented in the literature [99,101]. Furthermore conformational changes of the proteolipid system induced upon binding of different classes of ligands to the receptor have been monitored and showed that different classes of ligands produce distinctly different structures in the proteolipid system. Such studies are possible due to the potential of PWR to distinguish between changes in mass and changes in conformation occurring in anisotropic films which arises from the existence of an additional layer of silica on the sensor surface that functions as a waveguide, allowing polarization to occur with both p - (perpendicular) and s -

(parallel) polarized light. A detailed analysis of the principles of this technology has been given in the beginning of this review and elsewhere [9] and thus will not be further discussed here.

The different conformational states observed upon ligand binding to the receptor have been analyzed by fitting the PWR spectra to theoretical curves (obtained using Maxwell's equations) and the optical parameters extrapolated [99]. Such analysis have indicated that although both agonist binding and antagonist binding to the receptor cause increases in molecular ordering within the proteolipid membrane, only agonist binding induces an increase in thickness and molecular packing density of the membrane. This is interpreted as a consequence of mass movements perpendicular to the plane of the bilayer occurring within the lipid and receptor components. These results are consistent with models of receptor function that involve changes in the orientation of transmembrane helices and receptor elongation. A more simplistic rapid analysis is possible using graphical analysis [132]. This involves measurement of plasmon-waveguide resonance spectral shifts obtained upon molecular interactions occurring in deposited films with both *s*- and *p*-polarized excitation, and transforming these from an (*s-p*) coordinate system into a (mass-structure) coordinate system. This procedure has been illustrated by data obtained upon the binding of a small peptide, penetratin, to solid-supported lipid bilayer membranes [105].

Using a similar setup, the interaction of G-proteins with the hDOR bound to different types of ligands was also investigated, followed by the interaction of GTP γ S with the ligand-receptor-G-protein complex [100]. It should be pointed out that classic pharmacological methods only give indirect information about the interaction of GPCRs with G-proteins, because they are based on downstream responses. The GTP γ S assay reflects a combination of both the affinity of the G-protein to the ligand-bound receptor, and the ability of the agonist-bound state to initiate GDP dissociation from the G-protein. Thus, such measurements do not directly probe interactions between the various signal transduction partners and suffer the disadvantages of being very time consuming and dependent on the use of radiolabeled material. Another limitation of such experiments comes from the fact that cells usually express all the different G-protein subtypes making it very hard to understand the contribution of each individual subtype. Using PWR, the interaction of different G-protein subtypes ($G_{i\alpha1}$, $G_{i\alpha2}$, $G_{i\alpha3}$ and $G_{o\alpha}$) with hDOR, as well as the GTP γ S interactions with the G-protein-agonist-bound hDOR complex, was directly monitored and dissociation constants obtained for each of the separate processes. As can be seen in Table (3), there is a great degree of diversity and specificity in terms of the interactions of different ligand-activated receptor with different G-protein subtypes and there is no necessary correlation between the affinity of the G-protein to the ligand-receptor and its ability to undergo GDP/GTP exchange.

The fact that different ligands from the same class can lead to preferential interaction with certain G-protein subtypes, suggests that these preferences may lead to an additional source of drug side effects. Thus, certain ligands, by selecting specific G-protein subtypes over others may thereby be targeting specific signaling pathways that may lead to unwanted side effects. Alternatively, by designing ligands that activate only desired pathways, this might be avoided. PWR studies of the kind presented here may therefore have a great impact on drug screening protocols and design, as well as providing new insights into the basis for differential physiological and/or pharmacological effects of drug activity.

In the same studies, interactions of the individual α subunit and $\beta\gamma$ dimer were investigated showing that the presence of each subunit enhances the affinity of the other to the hDOR in a cooperative manner [102]. The high affinities obtained for the individual subunits to the agonist-activated receptor (especially for the β,γ subunits) (Table 4) suggests that the separated subunits may also have a biological function. There is evidence that G-proteins are differentially expressed in different tissues [133]. Furthermore, the α and $\beta\gamma$ subunits of the G-

protein interact with different effectors leading to different signal transduction events in the cell [134]. Considering the above, the fact that the subunits have the capability to act independently, with the α subunit even maintaining its catalytic activity in the absence of the $\beta\gamma$ subunits, may be essential for specific signalling pathways to be activated over others. G-protein subtypes can produce distinct metabolic effects. Elucidation of these specificities can thus have useful consequences.

FUTURE PERSPECTIVES

Surface Plasmon-based techniques offer a multitude of advantages for investigating various aspects of systems biology, and recently have been successfully applied to the investigation of GPCR signaling. Obvious advantages of this technology are label free, non-invasive, continuous and multispot capability of detection easily developed in a high throughput manner. There are an increasing number of commercially available instruments [78,135] that are driving forward the development of novel sensor surfaces, immobilization techniques and attachment chemistries allowing a great diversity of protein-ligand, protein-protein and protein-lipid interactions to be investigated. Scientists in both academia and industry are using optical biosensors in several aspects of drug discovery and signal transduction. The search for higher sensitivity and adaptation of this technology to high throughput strategies with rapid and high information content will continue over the next decade.

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Fig. (1).

Side view of a PWR experimental device containing a glass prism coated with a silver layer overcoated with a SiO_2 film. The thicknesses of the silver and silica film are 50 nm and 460 nm, respectively. Also shown is a deposited lipid bilayer held in place by a Teflon spacer via a plateau-Gibbs border. The prism, the detector and the aqueous compartment of the device are mounted onto a rotating table allowing adjustment of the incident light from 35 to 70 deg with 1 mdeg steps.

Fig. (2). Resonance spectra as a function of incident angle.

Panel A is for a 50 nm silver coated prism covered with 460 nm of silica. The dashed curve shows the SPR spectrum obtained without the protecting dielectric film. Panel B is for a similarly coated prism (50 nm silver, 50 nm TiO_2 and 750 nm low density dielectric film ($n = 1.35$)). Obtained from reference [9] with permission from the Biophysical Society.

Fig. (3). Amplitude of the electric fields.

Electric field amplitudes within a silver layer, a SiO₂ film, and a sensing layer for *p*- (A) and *s*- (B) polarized light, as a function of the distance from the glass-metal interface, for the design presented in Figure (1). Picture obtained from reference [9] with permission from the Biophysical Society.

Fig. (4).

(A) Schematic diagram of SPR imaging. **(B)** Calculated SPR reflectance curve for a pure gold surface (solid line), a reactant dot (dashed line) and adsorbed analyte molecules on a reactant dot (dotted line). **(C)** The contrast of the SPR image is based on the different reflectance $r_A > r_R > r_{Au}$. Picture obtained from reference [20] with permission from Springer Verlag.

Fig. (5).

Schematic representation of the immobilization of a histidine-tagged receptor to a NTA-functionalized sensor surface. (A) The chelator is covalently fixed to the support in two steps, first modifying the quartz surface by mercaptosilane in the gas phase followed by crosslinking NTA-maleimide in aqueous solution. (B) The receptor, in this case 5-HT₃, binds to the Ni²⁺ loaded NTA chelating groups via the histidine tag. Picture adapted from reference [69].

Fig. (6).
Schematic illustration of rhodopsin-transducin coupling on patterned supported membranes following a light flash. R and R* represent the inactive and active form of rhodopsin, respectively. Picture obtained from reference [69] with permission from Elsevier.

Fig. (7). Immobilization of the receptor.

Streptavidin binds to biotin-thiols in a mixed SAM and docks biotinylated receptor to the surface. (A) Carbohydrate-specific biotinylation of the latter results in uniform orientation of the bound receptor. The G-protein binds to the supported lipid bilayer, which is formed after receptor immobilization. (B) Photoisomerization of 11- cis- to all-trans-retinal triggers the active conformation of rhodopsin. G-protein bound to rhodopsin liberates its GDP and (C) desorbs from the membrane upon GTP binding, leading to a decrease in mass charge on the sensor chip that can be followed by SPR. (D) Active rhodopsin decays spontaneously to all-trans-retinal and opsin, and the G-protein binds upon nucleotide hydrolysis again to the surface. (E) 11-cis- and 9-cis-retinal both bind to opsin, regenerating photoactivatable rhodopsin. This figure was adapted from reference [136].

Table 1

List of the surfaces currently available from bia-core and affinity sensors

SPR surfaces Chemistry	General application
Biacore surfaces	
CM5 – carboxymethyl dextran	Routine analysis
SA-streptavidin	Biotin conjugation
NTA- nickel chelation	His-tagged conjugation
HPA-hydrophobic monolayer	Create hybrid lipid bilayers
B1- low charge	Reduces non-specific binding
C1-flat carboxymethylated	No dextran
F1- short dextran	Large analytes
J1- gold surface	User-defined surfaces
L1- lipophilic dextran	Capture liposomes
Affinity sensors surfaces	
CM-carboxymethyl dextran	Routine analysis
Hydrophobic planar	Create lipid mono layers
Amino planar	Alternative coupling chemistry
Carboxylate planar	No dextran
Biotinylated planar	Streptavidin conjugation

Note: this table was adapted from reference [78].

Table 2

Binding affinities between transducin (G_t) and rhodopsin (dark-adapted and light-activated) and between GTP γ S and transducin in DOPC and DOPC/DOPE (25:75) bilayers at pH 5^a

Bilayer Composition	K_D Dark-adapted		K_D Light-activated	
	DOPC	DOPC/DOPE (25:75)	DOPC	DOPC/DOPE (25:75)
G_t -Rhodopsin / nM	68 ± 9 [*]	64 ± 11 [*]	18 ± 3	0.7 ± 0.1
G_t -GTP γ S / nM			748 ± 85	736 ± 94

Note: this table was adapted from reference [98].

^aThe K_D values reported were obtained from plotting the resonance minimum position for the PWR spectra (Y) as a function of either G_t or GTP γ S concentration (X) and fitting the data to the following hyperbolic function that describes the 1:1 binding of a ligand to a receptor: $Y = (B_{max} \times X) / (K_D + X)$. Here B_{max} represents the maximum bound concentration and K_D is the concentration of ligand required to reach half-maximal binding. The K_D value represents the average of those obtained for *p*- and *s*-polarized light.

^{*} No PWR spectral changes were observed in this case.

Table 3

Dissociation constants between the individual G-protein subtypes (in the presence of a mixture of $\beta\gamma$ dimers) and the hDOR bound to DPDPE, deltorphin II, SNC80, tan67 or morphine and between GTP γ S and the ligand-receptor-G protein complex

G-protein subtype K _D G-protein(nM) K _D GTP γ S (nM)	DPDPE		
	G _{oα}	G _{ia1}	G _{ia3}
	10 ± 1 402 ± 54	304 ± 26 4.2 ± 0.5	43 ± 5 82 ± 10
G-protein subtype K _D G-protein(nM) K _D GTP γ S (nM)	Deltorphin II		
	G _{oα}	G _{ia1}	G _{ia3}
	4 ± 0.3 11 ± 1.2	58 ± 8 92 ± 11	125 ± 16 22 ± 6
G-protein subtype K _D G-protein(nM) K _D GTP γ S (nM)	SNC 80		
	G _{oα}	G _{ia1}	G _{ia3}
	5.0 ± 0.5 9.5 ± 1.8	215 ± 33 2.3 ± 0.4	18 ± 2 25 ± 3
G-protein subtype K _D G-protein(nM) K _D GTP γ S (nM)	(-)Tan 67		
	G _{oα}	G _{ia1}	G _{ia3}
	12 ± 1.2 8.5 ± 0.7	23 ± 1.7 75 ± 9	3 ± 0.2 5.4 ± 0.4
G-protein subtype K _D G-protein(nM) K _D GTP γ S (nM)	Morphine		
	G _{oα}	G _{ia1}	G _{ia3}
	43 ± 5 895 ± 128	34 ± 8 93 ± 10	17 ± 3 915 ± 145

Note: this table was adapted from reference [102].

K_D values were obtained from plotting the resonance minimum position for the PWR spectra as a function of G-protein/GTP γ S concentration and fitting to the following hyperbolic function that describes the binding of a ligand to a receptor: $Y = (B_{max} \times X) / (K_D + X)$. B_{max} represents the maximum concentration bound and K_D is the concentration of ligand required to reach half-maximal binding. Results presented were obtained from 3 independent experiments and standard deviations are presented.

* No PWR spectral shifts were obtained upon addition of GTP γ S up to 5 μ M.

Table 4
Dissociation constants between the $G_{i\alpha}$ subtypes or the $\beta\gamma$ dimer mixture and the hDOR bound to DPDPE, morphine or SNC80 and between GTP γ S and the receptor-G protein complex

Ligand	DPDPE		SNC80		Morphine	
G-protein subtype						
K _D G-protein(nM)	$G_{i\alpha 2}$ 213 ± 46		G_{oa} 282 ± 53		$G_{i\alpha 1}$ 352 ± 48	
K _D GTP γ S (nM)	90 ± 9		156 ± 29		910 ± 108	
	$\beta\gamma$ 44 ± 5 *		$\beta\gamma$ 26 ± 3 *		$\beta\gamma$ 49 ± 6 *	

Note: this table was adapted from reference [102].

* No PWR spectral shifts were obtained.

Results presented were obtained from 3 independent experiments and standard deviations are presented.