OPTICAL BIOSENSORS IN DRUG DISCOVERY

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Optical biosensors that exploit surface plasmon resonance, waveguides and resonant mirrors have been used widely over the past decade to analyse biomolecular interactions. These sensors allow the determination of the affinity and kinetics of a wide variety of molecular interactions in real time, without the need for a molecular tag or label. Advances in instrumentation and experimental design have led to the increasing application of optical biosensors in many areas of drug discovery, including target identification, ligand fishing, assay development, lead selection, early ADME and manufacturing quality control. This article reviews important advances in optical-biosensor instrumentation and applications, and also highlights some exciting developments, such as highly multiplexed optical-biosensor arrays.

BIOSENSOR
A device that uses biological receptors to detect analytes in a sample.

The analysis of molecular interactions is a key part of the drug discovery process; many millions of dollars are spent early in drug development on screening compounds for receptor binding *in vitro*. BIOSENSORS are commonly used for such tasks, and can give detailed information on the binding affinity, and in many cases also on the binding kinetics, of an interaction. Typically, the receptor molecule must be connected in some way to a sensor that can be monitored by a computer.

At present, most screens that are used in drug discovery require some type of fluorescent labelling or radiolabelling to report the binding of a ligand to its receptor. This labelling step imposes extra time and cost demands, and can in some cases interfere with the molecular interaction by occluding a binding site, which leads to false negatives. Fluorescent compounds are invariably hydrophobic, and in many screens, background binding is a significant problem, leading to false positives. Ideally, a biosensor-based screening platform should be label-free, sensitive and have sufficient throughput to be widely applicable in drug discovery.

The development of such a platform can be traced as far back as 1912, when R. M. Wood at Johns Hopkins University noticed that when he shone polarized light on a metal-backed diffraction grating, a pattern of unusual dark and light bands appeared in the background[1]. This effect was later described in terms of the excitation of electromagnetic 'evanescent' waves at the surface of the metal, and, in the 1970s, evanescent waves were described as a means to study ultra-thin metal films and coatings[2]. Finally, in the 1980s, surface plasmon resonance (SPR) and related techniques that exploited evanescent waves were applied to the interrogation of biological and chemical interactions[3–5]. These techniques allow the user to study the interaction between immobilized receptors and analytes in solution, in real time and without labelling of the analyte. Observed binding rates and binding levels can be interpreted in different ways to provide information on the specificity, kinetics and affinity of the interaction, or on the concentration of the analyte.

Since the development of the first commercial optical biosensor in the late 1980s, the use of optical biosensors in research and development has been described in more than 3,000 scientific publications that cover most disciplines in the pharmaceutical and diagnostic industries. These include ligand fishing[6,7], bacteriology[8–10], virology[11–13], epitope mapping[14–16], molecular engineering[17], cell biology[18–20], cell adhesion[21,22], signal transduction[23,24], nucleotide–nucleotide[25–27] and nucleotide–protein[28,29] binding, enzyme mechanisms[30,31] and so on. For more detailed information on the application of optical biosensors in the interrogation of intermolecular interactions in general, the reader is referred to recent comprehensive reviews[32–40].

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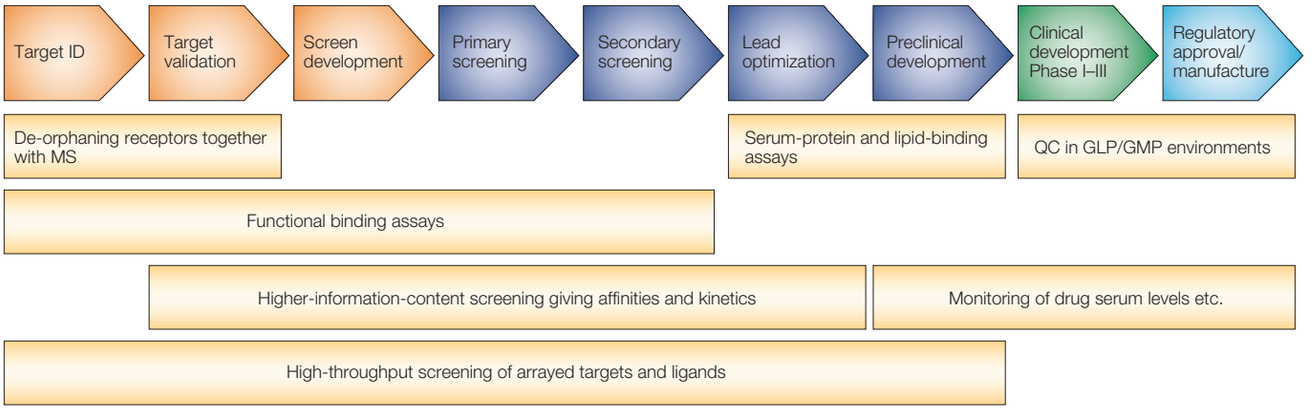


Figure 1 | **A simplified outline of the drug discovery process.** Application areas for optical biosensors are highlighted below the stages of the drug discovery process. GLP, Good Laboratory Practice; GMP, Good Manufacturing Practice; ID, identification; MS, mass spectrometry; QC, quality control.

Significant improvements in instrumentation and experimental design have allowed a wider variety of interactions to be studied in more detail, which has led to the increasing application of optical-biosensor technology throughout the drug discovery process (FIG. 1). For example, recent advances in instrument sensitivity have enabled the direct detection of small-molecule binding to immobilized receptors, which has greatly increased the utility of optical biosensors in drug screening. Several manufacturers now offer optical biosensors that are tailored to various applications (TABLE 1 and online TABLE 1). This review, along with the accompanying information online, describes some of the underlying technology of optical biosensors, and then highlights their use in key areas of drug discovery.

Biosensor technology

Optical biosensors exploit the EVANESCENT-WAVE PHENOMENON to characterize interactions between 'receptors' that are attached to the biosensor surface and 'ligands' that are in solution above the surface. Many of the best-known optical biosensors use SPR, for which a typical experimental set-up is shown in FIG. 2. Binding of molecules in solution to surface-immobilized receptors alters the refractive index of the medium near the surface. This change can be monitored in real time to measure accurately the amount of bound analyte, its affinity for the receptor and the association and dissociation kinetics of the interaction (FIG. 3). An extremely wide range of molecules can be analysed, from low-molecular-mass drugs to multiprotein complexes and bacteriophage, with interaction affinities from millimolar to picomolar in strength. Most importantly, binding affinities and kinetics can be determined using very low amounts of compound without the need for prior chemical labelling or radiolabelling.

EVANESCENT-WAVE PHENOMENON
Total internal reflection of light at a surface–solution interface produces an electromagnetic field, or evanescent wave, that extends a short distance (~100–200 nm) into the solution. SPR is an evanescent-wave phenomenon that occurs at certain metallic surfaces.

Table 1 | **Manufacturers of optical-biosensor systems**

Manufacturer	Web address	Technology
Affinity Sensors	www.affinity-sensors.com/	Resonant mirror
Artificial Sensing Instruments	www.microvacuum.com/research/memocs/	Waveguide
Aviv Instruments	www.avivinst.com/	Grating-coupled SPR
Biacore	www.biacore.com/	SPR
Farfield Sensors	www.farfield-sensors.com/	Waveguide
Graffinity Pharmaceuticals*	www.graffinity.com/	SPR
HTS Biosystems	www.htsbiosystems.com/	Grating-coupled SPR
IBIS	www.ibis-spr.nl/	SPR
Luna Analytics	www.lunaanalytics.com/	Grating-coupled fibre optic
Nippon Lasers	www.rikei.com/spr	SPR
Prolinx	www.prolinx.com	SPR
SRU Biosystems	www.srubiosystems.com/	Guided-mode resonant filter

*Graffinity Pharmaceuticals does not sell instrumentation, but can, on request, screen targets against surface-immobilized small molecules using its Plasmon Imager array system. SPR, surface plasmon resonance.

The interface between the sensor surface and the chemical or biological systems to be studied is a key component of optical biosensors. Receptors must be attached to some form of solid support, while retaining their native conformation and binding activity. This attachment must be stable over the course of a binding assay, and in addition, sufficient binding sites should be presented to the solution phase to interact 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 (for example, a gold layer) and the biological component to achieve these ends. Functionalized alkane thiols[41] and alkoxy silanes[42] form stable, self-assembled monolayers on planar surfaces and act as ideal linkers. The alkyl termini of these molecules can be derivatized with ethyleneglycol subunits to produce a planar surface that is resistant to the non-specific adsorption of proteins[43], or can be mixed with molecules that have suitable chemical reactivity for receptor capture (for example, epoxy, carboxyl, amino, biotinyl, nitrilo triacetic-acid groups)[43–45]. The larger binding partner (for example, the protein target) is normally immobilized on the surface, and the smaller binding partner (for example, the drug) is allowed to bind to this surface from free solution. However, in some cases, drug-like molecules

have been attached directly to the chemical-linker layer and receptors have been passed over the surface[46,47]. Graffinity Pharmaceuticals has taken this approach to challenge the dogma that small molecules must always be screened in free solution. Here, chemical compounds from a library fitted with a molecular tag are spotted onto a gold surface that is decorated with a protein-resistant linker layer. The resultant chemical microarray is then exposed to the target protein, and the extent of binding to each spot in the array is determined simultaneously using wavelength-mode SPR (see below).

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 adsorption of proteins, nucleotides and drugs. The same polymer also provides a three-dimensional scaffold for receptor immobilization. The most widely used biosensor polymer coat is carboxymethyl dextran[48], although other materials that produce a protein-resistant hydrogel can also be used, such as hyaluronic acid, polyvinyl alcohol, polymethylmethacrylate, sepharose and so on. There are many strategies for either covalent (BOX 1) or non-covalent (BOX 2) attachment of receptors to either planar self-assembled surfaces or polymer coats. Selection of the correct coupling chemistry requires careful consideration of the resultant orientation of the receptor, 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. Membrane proteins present their own unique challenges and are dealt with later in this article.

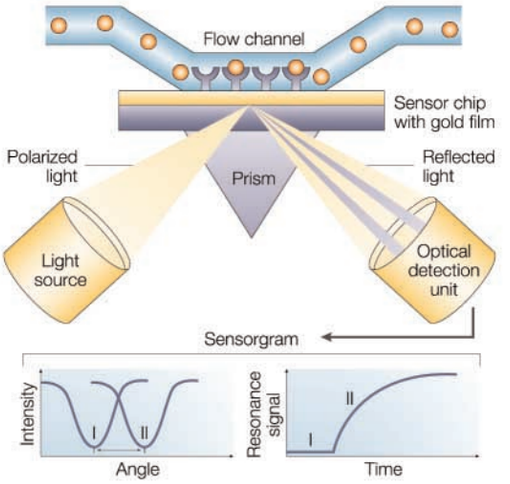


Figure 2 | **Typical set-up for an SPR biosensor.** Surface plasmon resonance (SPR) detects changes in the refractive index in the immediate vicinity of the surface layer of a sensor chip. SPR is observed as a sharp shadow in the reflected light from the surface at an angle that is dependent on the mass of material at the surface. The SPR angle shifts (from I to II in the lower left-hand diagram) when biomolecules bind to the surface and change the mass of the surface layer. This change in resonant angle can be monitored non-invasively in real time as a plot of resonance signal (proportional to mass change) versus time.

Target characterization

Optical biosensors are often viewed solely as a tool for intricate, low-throughput, kinetic analysis of binding events, and are used to answer the question: how fast does an analyte associate with, and dissociate from, its receptor? However, in addition to this important information about the kinetics of an interaction, optical biosensors can also be used to address more basic questions, such as: Does the analyte bind at all? How strong is the binding? How much of the sample is active? Qualitative information on protein binding can also be used to deconvolute complex biochemical pathways and identify key binding subunits in multiprotein complexes[24,49]. An information-rich assay that gives a quantitative ranking of interaction affinities and the active concentration of an expressed receptor or ligand can be extremely valuable in the early stages of drug discovery.

Ligand fishing. In receptor-driven drug discovery, crude tissue extracts and cell homogenates are screened for potential ligands of orphan receptors — an approach that is often termed 'ligand fishing'. Once a positive-binding cell-line-conditioned medium or cell homogenate has been identified, the rate-limiting step is then the purification of the ligand for amino-acid sequencing. This normally involves the production of large volumes of conditioned media, followed by receptor affinity chromatography and concentration. This can be problematic when ligands are discovered in body fluids of limited volume, such as synovial fluid[50]. Here, an optical biosensor can be used as a micro-preparative affinity-purification device[7], which screens for binding activity and simultaneously concentrates the ligand for identification by tandem electrospray mass spectrometry or other methods (see below)[51,52].

'Hit' confirmation. At present, optical biosensors are used in drug discovery primarily to confirm high-throughput screening (HTS) 'hits' from fluorescence-, chemiluminescent- or radiometric-based screens. Once a receptor–analyte binding pair has been identified, biosensors can then rapidly screen a variety of buffers and regeneration conditions to optimize binding efficiency. Hence, they can be used not only for rapid secondary screening of novel compounds, but also to accelerate the *in vitro* assay development that facilitates elucidation of the mode of action of a putative drug. The analysis of interaction affinities and kinetics has been facilitated by improvements in experimental design (BOX 3), data analysis and software[53–55].

Monitoring antibody and cytokine production. Optical-biosensor measurements can be made continuously in real time, and are hence ideally suited for online monitoring of antibody and cytokine production, often with greater precision than more traditional end-point assays, such as enzyme-linked immunosorbent assays (ELISAs) and western blotting. In this application, the response at varying concentrations of analyte is plotted to produce a calibration curve that is used to analyse unknown samples[56,57].

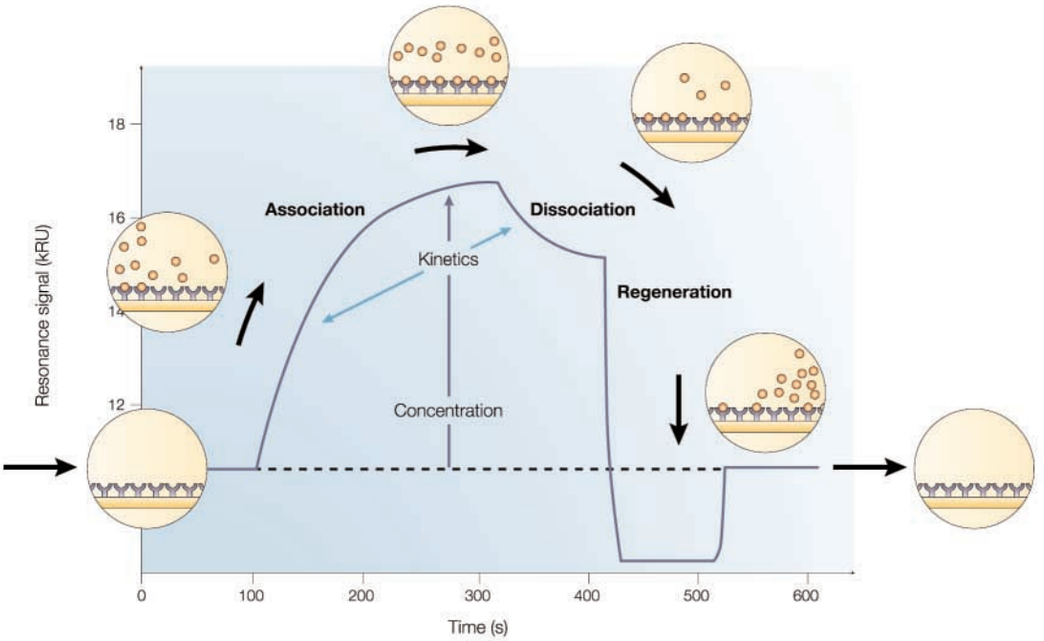


Figure 3 | **A typical binding cycle observed with an optical biosensor.** A molecule is immobilized on the sensor surface with appropriate coupling chemistry. At t = 0 s, buffer is contacted with the receptor through a microfluidic flow cell, or in some commercial instruments, through a cuvette. At t = 100 s, a solution of analyte in the running buffer is passed over the receptor. As the analyte binds to the surface, the refractive index of the medium adjacent to the sensor surface increases, which leads to an increase in the resonance signal. Analysis of this part of the binding curve gives the observed association rate (k_{obs}). If the concentration of the analyte is known, then the association rate constant of the interaction (k_{ass}) can be determined. At equilibrium, by definition, the amount of analyte that is associating and dissociating with the receptor is equal. The response level at equilibrium is related to the concentration of active analyte in the sample. At t = 320 s, the analyte solution is replaced by buffer, and the receptor–analyte complex is allowed to dissociate. Analysis of these data gives the dissociation rate constant (k_{diss}) for the interaction. Many complexes in biology have considerable half-lives, so a pulse of a regeneration solution (for example, high salt or low pH) is used at t = 420 s to disrupt binding and regenerate the free receptor. The entire binding cycle is normally repeated several times at varying concentrations of analyte to generate a robust data set for global fitting to an appropriate binding algorithm. The affinity of the interaction can be calculated from the ratio of the rate constants ($K_D = 1/K_A = k_{diss}/k_{ass}$) or by a linear or nonlinear fitting of the response at equilibrium at varying concentrations of analyte. In addition to determining the interaction affinities and kinetics, a thermodynamic analysis of a biomolecular interaction is also possible. This is done by applying van't Hoff's equations to the interaction affinities and kinetics of an interaction obtained at various different temperatures[116,117].

Compound screening and lead optimization

The identification of an appropriate lead structure is a key step in drug discovery. Some drug discovery companies are shifting away from ultra-HTS (uHTS) towards more focused screening of drugs and drug fragments. In this arena, optical biosensors are finally gaining acceptance as high-information-content screening tools.

The first commercially available optical biosensors had limited sensitivity and could reliably detect only the binding of larger molecules, such as proteins, to the surface. In 1994, a surface-competition assay format was developed that allowed indirect detection of small-molecule binding[58]. In this format, the target receptor is immobilized on the surface as usual. The small-molecular-mass compound to be screened is then mixed together with an antibody that is directed against the receptor-binding site, or with a protein–drug conjugate that binds to the receptor. The concentration of the antibody or protein conjugate is kept constant so that changes in the observed response are proportional to the amount of small-molecular-mass compound that is bound to the receptor on the surface. This solution-competition approach provided a workable format for drug screening; however, the assay development that was required was generally not compatible with HTS.

The application of optical biosensors to drug screening only really gained acceptance when technical advances in instrumentation sensitivity and software allowed the binding of small molecules to be detected directly, and the resultant data to be archived and integrated with knowledge-management systems. With molecular-mass sensitivity in the order of 200 Da, a direct-binding assay can be used, in which the target receptor is immobilized and small-molecular-mass compounds are injected over the surface. This direct-screening approach has been applied to the selection of thrombin inhibitors[59], HIV-protease inhibitors[60,61], DNA-gyrase inhibitors[62] and many others. Quantitative kinetics of compound binding can be used to gain a higher level of understanding about binding mechanisms, as it is possible to investigate the effect of structural variations in a systematic way. Association and dissociation rates can be varied independently for a specific lead series, resulting in the rapid evolution of subnanomolar-affinity leads[60].

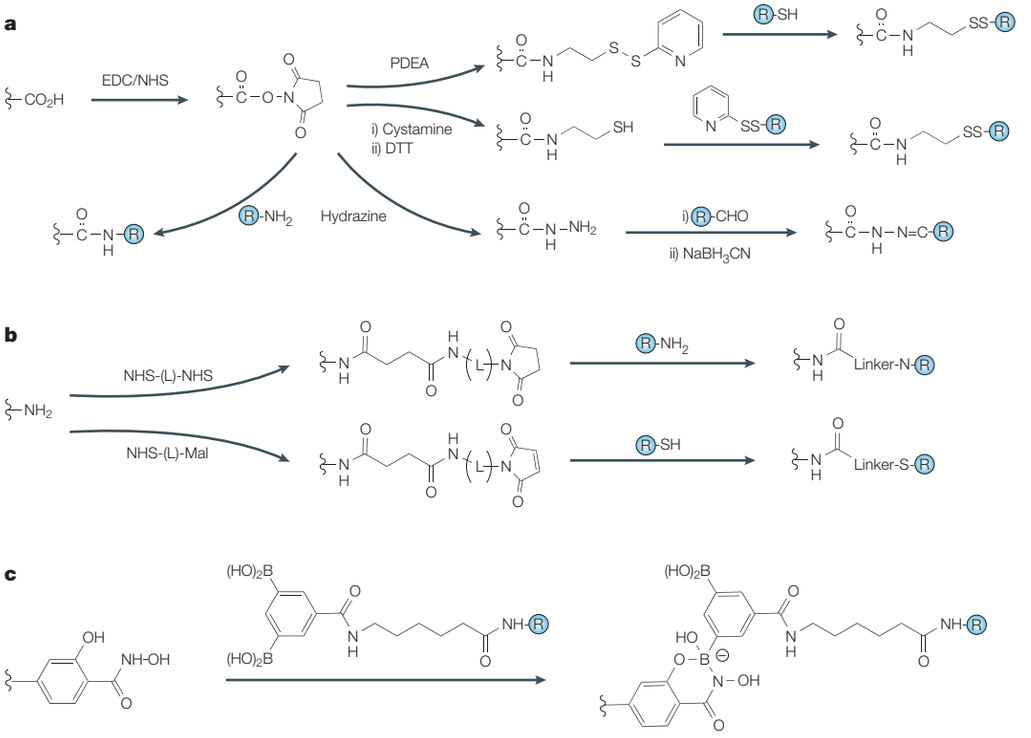
A direct small-molecule binding assay is well suited to confirmatory screening or validation of hits from primary screens, and to lead optimization. The resultant data identify binders from a single sample concentration and are information rich, which allows ranking of both binding-complex affinity and stability by using both the equilibrium and dissociation phases (FIG. 4a). In particular, the unique kinetic information on the binding stability that is extracted from the dissociation-phase data can be used to rank leads during optimization of drug properties[61]. Simple inspection of the data in FIG. 4a shows clear differences in the binding levels and dissociation rates for

Box 1 | **Coupling methods for receptor immobilization: covalent attachment**

Immobilization of a receptor to the sensor surface is of central importance to the design of a successful biosensor assay[53]. The coupling method must be efficient, produce a highly stable association (to prevent signal drift) and allow control of the amount of receptor that is immobilized. Amine coupling (for example, to the amino terminus or surface lysine residues on a protein) will lead to a heterogeneous population of receptors with random orientation on the surface. Affinity-capture (BOX 2) and sulphydryl couplings can be used to produce a more homogeneous population of oriented receptors on the surface[102–104]. Strategies that are commonly used to covalently attach a receptor to a surface include:

- Water-soluble EDC-mediated activation of a carboxymethylated support, such as dextran or hyaluronic acid (panel **a**). The resultant reactive NHS ester can then be coupled directly with available amino moieties of a receptor (R) to form a stable amide linkage. Acidic receptors (with an isoelectric point (pI) <3.5) are difficult to immobilize by amine coupling, as the low pH that is required for electrostatic pre-concentration to the sensor surface protonates the primary amino groups and reduces the coupling efficiency. Further derivatization with sulphydryl-reactive reagents (for example, PDEA or SPDP) allows reaction with free surface thiols (for example, cysteine or methionine) to form a reversible disulphide linkage. In a similar manner, stable thioether bonds can be formed using maleimide coupling reagents, such as sulpho-SMCC and GMBS. The surface can also be derivatized with cystamine to effect coupling with disulphide-activated receptors. Finally, treatment with hydrazine followed by a reductive amination allows coupling with aldheydes. The aldehyde groups could be native to the receptor or formed by mild oxidation of any *cis*-diols that are present.
- Amino-presenting surfaces[45,105] can be treated with commercially available bifunctional linking reagents to effect coupling with free amino or sulphydryl groups on the receptor (panel **b**).
- Surfaces that are derivatized with SHA can be used to produce reversible complexes with receptors that have been activated with PDBA[106] (panel **c**):

DTT, dithiothreitol; EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; GMBS, *N*-(γ-maleimidobutyrloxy) sulphosuccinimide ester; L, linker; Mal, maleimide; NHS, *N*-hydroxysuccinamidyl; PDBA, phenyldiboronic acid; PDEA, pyridinyldithioethanamine; SHA, salicylhydroxamic acid; SPDP, 3-(2-pyridinyldithio)propioic acid *N*-hydroxy-succinimide ester); sulpho-SMCC, sulphosuccinimidyl-4-(*N*-maleimidomethyl)cyclohexanecarboxylate.



Box 2 | **Coupling methods for receptor immobilization: non-covalent attachment**

Biotin- or streptavidin-presenting surfaces

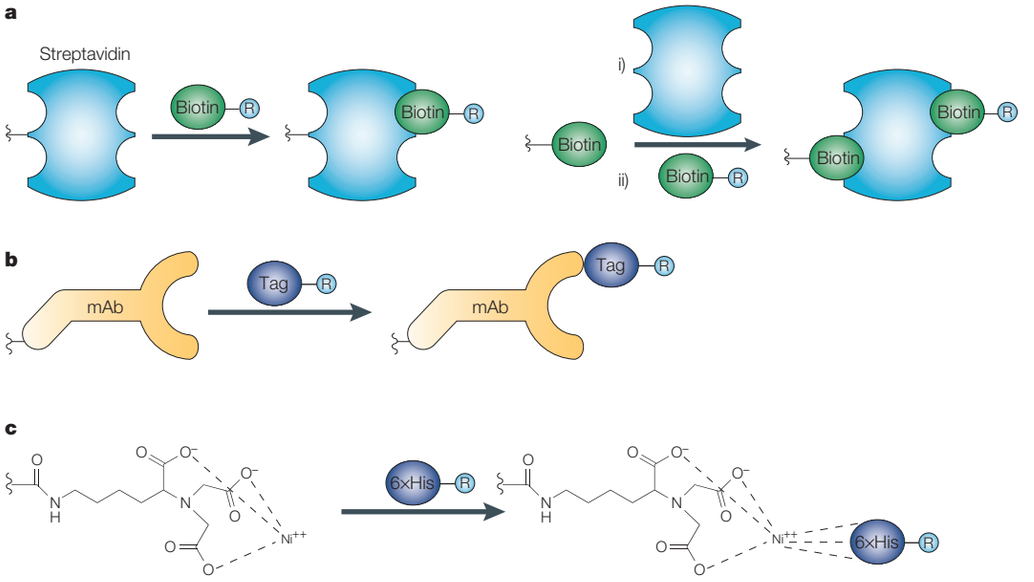
These can be used to capture biotinylated-receptors (panel **a**). The multiple BIOTIN-binding sites of streptavidin on each face of the molecule allow biotinylated ligands to be crosslinked by the streptavidin 'double adaptor'. This method is highly efficient and leads to stable complexes, but is effectively irreversible. It is commonly used to immobilize 5′-biotinylated oligonucleotides[25,28,107,108].

Monoclonal antibodies

These can be covalently attached to a solid support by means of amine coupling as in BOX 1a. Epitope-tagged or fusion proteins can then be directly and reversibly coupled to the surface through the antibody–antigen interaction[53,109,110] (panel **b**). Commonly used tags include, for example, glutathione *S*-transferase, herpes simplex virus glycoprotein D epiptope, FLAG epitope and 6 × His.

Metal-coordinating groups

Groups such as iminodiacetic acid (IDA) and nitrilotriacetic acid (NTA) have been widely used for direct immobilization of 6 × His- and 10 × His-tagged receptors[44,111–113] (panel **c**). The moderate affinity of the chelate–Ni^{2+}–histidine ternary interaction means that there is sometimes considerable decay in the level of immobilized receptor. For this reason, anti-6 × His monoclonal antibodies are often used to enable stable, oriented immobilization of His-tagged receptors[114].



the different compounds. After an initial threshold screen that was made simply on the basis of binding level, those compounds with low responses can quickly be discarded. This analysis identifies and confirms compound 'hits', and allows an initial ranking of binders. Plotting the equilibrium-binding level versus the binding-stability level can help to assess binding efficiency (FIG. 4b). Stable binders have slow dissociation rates and can be clearly separated from rapidly dissociating and non-binding compounds, as shown in FIG. 4b. Analysis of the association phase can give insight into the recognition mechanisms and QSARs (quantitative structure–activity relationships) that predicate rational drug design. In addition, by using multiple surfaces or spots with multiple receptors, it is possible to quality control for non-specific binding to non-target receptors. It is notable that in a recently published screen of peptides for binding to the HER2 tyrosine-kinase receptor (which is present in ~30% of breast and ovarian cancers), a strong correlation was found between the peptide-binding characteristics that were determined by SPR and their biological activity. In particular, dissociation rate constants for peptide binding to the receptor were shown to be better indicators of biological activity *in vivo* than simple binding affinities[63].

Integration with 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 two-dimensional 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

BIOTIN
The streptavidin/biotin system has one of the largest free energies of association observed for noncovalent binding of a protein and small ligand in aqueous solution (K_D = 0.1 pM). The complexes are also extremely stable over a wide range of temperature and pH.

Box 3 | **Correcting for mass transport and bulk effects**

Optical biosensors generally require a surface-immobilized receptor to function. It is therefore 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 re-binding of the analyte in the dissociation phase, as the released analyte can re-bind 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 analytes 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 has the effect of reducing the depth of the surface-associated 'unstirred' layer[53]. It is also possible to introduce a 'mass transport' rate constant into fitting algorithms to ensure that the binding data are correctly analysed[54].

Results using a computer simulation led to the suggestion that the carboxymethyl-dextran hydrogel that is most commonly used with optical biosensors could significantly retard the diffusion of analyte to its receptor at the surface[115]. 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[53].

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 leads 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[53]. This does not normally affect assays that are carried out in biological buffers, as bulk refractive changes can be corrected for successfully by subtraction of a reference surface that contains no receptor, or a non-relevant control receptor. However, small-molecular-mass compounds are invariably 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[72].

also their potential interactions with each other. The integration of mass spectrometry with optical-biosensor detection and separation can provide valuable extra information about target function and binding specificity, which can accelerate the development of novel drugs that target the receptor[52].

Early attempts at such integration involved the immobilization of a receptor on the biosensor surface that was used to capture analyte from a complex mixture. The identity of the captured analyte was carried out by 'off-chip' MALDI–TOF[64,65]. For example, epitope-tagged peptides have been detected and identified directly from *Escherichia coli* lysates at low-femtomole to sub-femtomole levels[66], as have ligands that bind to the receptor that is necessary for pollen attachment to plant stigma[67]. Unfortunately, this approach has several limitations: sequence analysis is limited in MALDI–TOF, which is not suitable for analysis of very large or very hydrophobic proteins; the preparation of the sensor chip for MALDI–TOF requires several handling steps; the process destroys the sensor surface, which prevents further processing and analysis; and finally, many proteins are post-translationally modified by enzymatic cleavage, phosphorylation, glycosylation and so on, which makes identification on the basis of molecular mass alone much more difficult.

To overcome these limitations, methodology was developed to elute the captured analyte from the sensor chip *in situ* in the biosensor instrument[68,69]. This approach also 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[51], identification of ligands that bind to orphan G-protein-coupled receptors (GPCRs) using immobilized mammalian-cell membrane fragments[70], the analysis of Staphylococcal toxins in food[71], and the identification of endoproteases using immobilized, affinity-tagged recombinant proteins with unique protease cleavage sites[30]. As the level of investment by pharmaceutical and biotechnology companies in proteomics research increases, the use of optical biosensors for the identification of proteins is likely to increase.

Early ADME

The extent to which drugs bind serum protein is an essential factor that must be considered when determining drug pharmacokinetic and activity profiles. Many compounds bind reversibly to human serum albumin (HSA), α_1-acid glycoprotein (AGP) and other serum components, such as immunoglobulins. A high level of protein binding reduces the free drug concentration and hence the physiological activity of the drug. Circulating protein–drug complexes also serve to replenish the free drug concentration, and thereby

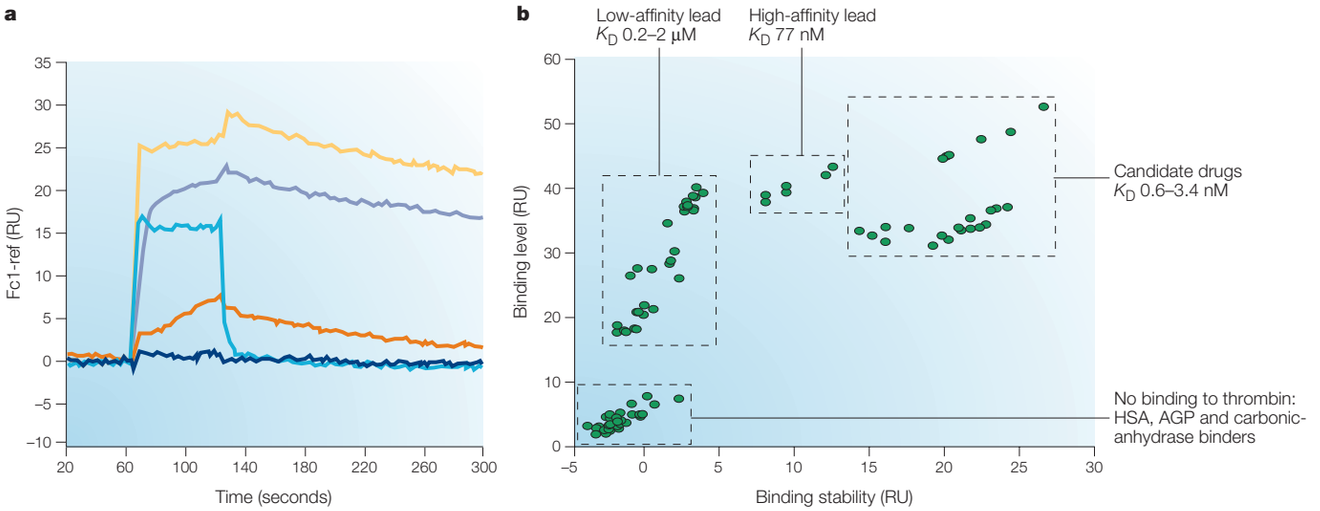


Figure 4 | **Examples of direct drug-screening assay using an optical biosensor.** In a direct binding assay, the drug target is immobilized to a sensor surface, and the interaction with low-molecular-mass binders is monitored directly[61]. The screen is run in duplicate in random order using known binders and non-relevant compounds as positive and negative controls, respectively. **a** | Some example traces from a screen in which human immunodeficiency virus (HIV)-1 protease was immobilized on a carboxymethyl-dextran surface, and proprietary compounds were passed over the receptor with automatic in-line reference subtraction of data from a reference surface that contains no receptor. The reference surface is needed to correct for bulk refractive index changes and signal drift, and to control for non-specific binding. Orange trace: lead with slow on- and slow off-rates; K_D = 36 μm. Turquoise trace: lead with high on- and high off-rates; K_D = 1 μm. Lilac trace: optimised lead with high on- and slow off-rates obtained by combining structural features of leads with orange and turquoise traces; K_D = 27 nm. Yellow trace: reference drug (ritanovir) with high on- and slow off-rates; K_D = 22 nm. Dark blue trace: negative control (sulfadimethazine). **b** | Thrombin was immobilized on a carboxymethyl-dextran surface and proprietary compounds assayed as above[59]. Carbonic anyhdrase, human serum albumin (HSA) and α_1-acid glycoprotein (AGP) were included as negative controls. The thrombin binding level attained at equilibrium during the association phase (a measure of binding affinity) was then plotted against the level reached at a set time during the dissociation phase (a measure of binding stability). Panel **a** adapted from REF. 61 © (2000), with permission from Liebert Online Electronic Journals. Fc1-ref, reference surface (blank); RU, resonance unit.

prolong the duration of drug action. Hence, the level of protein binding is an important factor in the delicate balance between intended physiological activity, long-term efficacy and potential side effects of the drug[72]. When large numbers of compounds need to be screened, it is convenient to use purified serum proteins rather than blood plasma or serum. This allows the extent of protein binding to be probed at an earlier stage in the drug discovery process, which helps to eliminate compounds with potentially poor ADME (absorption, distribution, metabolism and excretion) properties soon after HTS. Optical biosensors are ideally suited to be an alternative to more traditional assays, such as equilibrium dialysis or affinity chromatography, because sample consumption is low, the assay is rapid and — with automated systems — throughput is high. Early ADME characterization of a lead series using optical biosensors therefore allows the simultaneous optimization of drug–target interactions and ADME properties. Different serum proteins can be immobilized on different surfaces, and the affinity of a compound for the protein can be calculated by analysing the amount bound at varying compound concentrations. Knowing the concentration of the protein in serum, it is then possible to convert the calculated affinity to a more meaningful 'percent compound bound'. In addition, the half-life ($t_{1/2}$) of drug residence on serum proteins can be determined, which gives important information about compound turnover. This type of approach has been applied to 100 different drugs, using less than 100 μl of each compound at a concentration of 80 μM. An excellent correlation was found between the values for percent bound to serum proteins that were determined using an optical biosensor, and those data obtained using conventional methods[72]. The correlation was notably weaker for those compounds that were more than 97% protein bound.

In addition to serum binding, a key ADME parameter that must be assessed in the drug discovery process is the extent of passive and active adsorption of a drug to membrane interfaces in the gastrointestinal tract and blood–brain barrier. Applications of optical-biosensor technology in this area are still being developed, but early work has shown that immobilized lipid vesicles or cell membranes can be combined with SPR[73] and grating-coupler[74] detection to determine the affinity of drugs for these membranes. The resultant affinity ranking gives an indication of the fraction of drug that is passively adsorbed, and correlates well with data obtained by more traditional methods. Other assays are being developed to monitor the extent of drug binding to metabolic enzymes, such as glutathione *S*-transferases and cytochrome P450s, to enhance the *in vitro* early ADME profile using optical biosensors.

Quality assurance and control

Adherence to the regulations of Good Laboratory Practice (GLP) and Good Manufacturing Practice (GMP) is a legal requirement for manufacturers of diagnostic products and pharmaceuticals. Furthermore, FDA directives require a validated binding assay as part of the product-release portfolio for all therapeutic antibodies. Traditional biological assays for drug response, which use *in vitro* cell cultures or *in vivo* animal models, are expensive and not always reliable. Provided that there are sufficient physicochemical data for drug response (receptor binding does not always correlate with drug potency), binding assays that use purified receptors or membrane fragments are an accepted alternative[75,76]. In this demanding environment, the successful integration of automated instrumentation with validated software, data management and training procedures is of paramount importance.

Optical biosensors have not received widespread application in this area; however, there are several recent references that highlight the potential of the technology in the analysis of clinical samples, in particular in the serological analysis of antibody titres and affinities[77–79]. To exploit these applications, one biosensor manufacturer has recently released a dedicated instrument (Biacore C; developed by Biacore) for concentration analysis of biopharmaceuticals and vaccines within a validated GMP/GLP environment. Optical-biosensor assays have several advantages over traditional *in vitro* techniques, such as ELISA, high-performance liquid chromatography (HPLC), fast-performance liquid chromatography (FPLC) and NEPHELOMETRY — results are available with minimal sample preparation, usually within a few minutes, and with high accuracy and precision (claimed coefficients of variation are in the order of 5%). Instrument software and qualification that conforms to FDA regulation 21 CFR part 11 (which governs data integrity and multiple access levels) is now available. As accuracy and rapid assay turnover can be crucial for process development and fermentation monitoring, it is likely that optical biosensors will have an increasingly important role in these areas.

Screening against membrane 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 half of the 100 best-selling drugs on the market are targeted to membrane receptors. 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[70,80,81]. 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, and it also does not allow the study of signalling cascades that are triggered by ligand binding to a receptor, 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[82]. However, interactions with membrane receptors are surface-related processes that are difficult to study with bulk techniques. 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.

Supported lipid monolayers. The simplest method for the immobilization of membranes on a sensor surface is simply to adsorb the lipid onto a hydrophobic surface[83]. This results in the formation of a supported lipid monolayer, in which the hydrophobic acyl chains of the lipids contact the hydrophobic surface, and the polar lipid head groups are presented to solution (FIG. 5a). This method is generally limited to receptors that are anchored only in the outer leaflet of a native membrane, or to cases in which the analyte binds to the lipid itself. Lipid monolayers are normally formed from small, unilamellar vesicles, which spontaneously adhere to a hydrophobic self-assembled monolayer (SAM) with concomitant release of their strain energy. 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 GM_1[83] — a receptor–ligand pair that has been used widely to validate model membrane systems[84,85].

Tethered lipid bilayers. A lipid layer that is directly adsorbed onto a surface has the significant drawback that it cannot accommodate transmembrane proteins with sizeable 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 (FIG. 5b). These 'tethered bilayer membranes' (tBLMs) are attached in various ways to a solid support. They are readily formed by self-assembly, are very stable, and can be probed not only by SPR, surface plasmon fluorescence spectroscopy (SPFS), resonant-mirror and waveguide techniques[86], but can also be analysed with electrical measurements if the surface is conducting (for example, metals, indium–tin oxide and conducting polymers). Much of the pioneering work in this area has been carried out by Vogel

NEPHELOMETRY
The measurement of solution turbidity or 'cloudiness'. It can be used to study drug solubility and microbial growth, and for immunological tests.

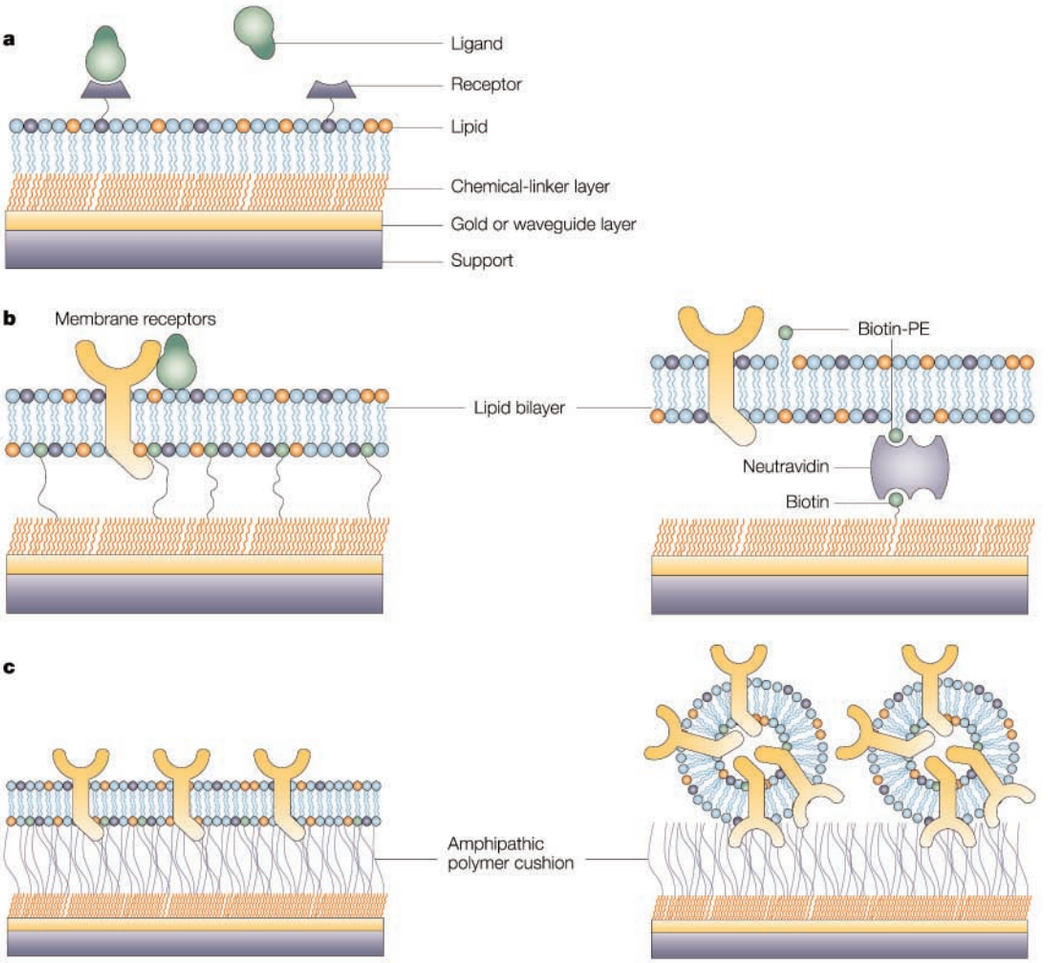


Figure 5 | **Model membrane systems that are used with optical biosensors. a** | A supported lipid monolayer that has been formed on top of a hydrophobic, self-assembled monolayer on a gold surface or a waveguide layer. **b** | Two examples of tethered lipid bilayers that contain an integral (transmembrane) receptor. The bilayer is either captured on the surface using synthetic phospholipids that are tethered to the support by flexible, hydrophilic linkers (left), or through immobilized neutravidin in conjunction with biotinylated lipids or a biotinylated receptor. **c** | Flexible, amphipathic polymer cushions support membranes as either supported lipid bilayers or captured proteoliposome layers. PE, biotinyl-phosphoethanolamine-*N*-(biotinyl).

and co-workers, who used a thiophospholipid that has a triethyleneglycol spacer unit to capture membranes and membrane proteins[87]. This approach has enabled the functional reconstitution of membrane proteins such as rhodopsin[45,80], myristoylated alanine-rich C kinase substrate[86], outer membrane protein F[88] and nicotinic-acetylcholine receptors[89]. A similar strategy has been used by Cornell and co-workers, with the addition of a membrane-spanning thiophospholipid that greatly improved the stability of the tBLM[90]. A Teflon spacer has been used by Tollin and co-workers to support bilayers over a silver film to allow SPR detection of transducin binding to the GPCR rhodopsin, and also to probe the interaction of cytochrome *c* with bovine-cytochrome-*c*-oxidase- and cardiolipin-containing membranes[91,92].

Polymer-supported bilayers. To overcome problems associated with roughness of the underlying surface, lipid bilayers can be bound to, but structurally de-coupled from, the solid support, by first coating it with a flexible polymer (FIG. 5c). These soft polymer cushions provide a hydrated lubricating layer between the surface and the membrane that allows the 'self-sealing' of surface defects. In this manner, the polymer cushion resembles the cytoskeleton that anchors the plasma membrane of a cell. 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[93]; coupling of lipopolymers that have functionalized hydrophobic head groups to a surface[94]; or the use of co-polymers 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[95,96]. 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'[70,97].

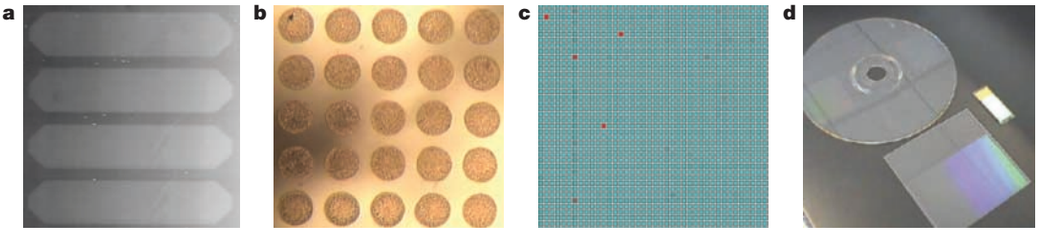


Figure 6 | **Examples of arrayed SPR detection. a** | The four-channel flow cell that was pioneered by Biacore within an area of 2.4 mm × 2.6 mm. **b** | Schematic representation of a Biacore functional array that is under development at present — 25 spots in a 5 mm × 5 mm area. The array will provide functional information on the protein-binding patterns and generate detailed kinetic data, in addition to concentration and affinity measurements. **c** | An image taken with the Plasmon Imager from Graffinity Pharmaceuticals of part of an array of 4,089 spots in an area of 80 × 80 mm. The figure shows a schematic representation of the binding profile of a protein against 4,608 compounds, each immobilized on a different field of a sensor plate. The surface plasmon resonance (SPR) wavelength shifts are detected after addition of 200 nM of protein. Larger wavelength shifts indicate stronger binding. **d** | HTS Biosystems' plastic optical diffraction gratings that are now under development, which could support a surface density of 10,000 spots in an area of 10 × 10 mm. The plastic chips are produced at low cost with the same methods that are used to manufacture CD-ROMs and DVDs, which allows for a great deal of flexibility in format design.

Microarrayed lipid layers. With the numerous orphan GPCRs and other novel membrane-receptor targets that have now 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[98] and Cremer[99]. Both groups used a process called micro-contact printing (μCP), 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[100] have formed mixed, self-assembled monolayers of hydroxyl- and cholesterol-terminating thiols to capture lipid bilayers, which can be microarrayed using micro-contact printing techniques.

Optical-biosensor arrays

The sequencing of the entire genome of many organisms, including humans, has had a great impact on the field of genomics. Accurate annotation of genetic sequences has provided the impetus for the rapid development of methods to analyse the composition and function of organism proteomes. Many pharmaceutical companies invest heavily in programmes that are based on genomic technologies; namely DNA sequencing, mutation/polymorphism detection and expression monitoring of messenger RNA. Because the ultimate targets for these programmes are actually proteins, more and more emphasis has been placed on protein-based methods in an effort to define the function of proteins that are discovered by genomic technologies[70]. The analysis of many complex binding events requires multiplexed detection systems that can analyse many binding interactions simultaneously. Commercially available optical biosensors have been limited in their application to HTS and proteomics analysis by the low number of surfaces or spots that could be sensed simultaneously (FIG. 6a). A key breakthrough was achieved in 1988 by Rothenhausler and Knoll[101], when they showed the simultaneous imaging of an entire surface using surface plasmon microscopy (SPM), which has similar basic principles to SPR.

As a result of the emerging commercial potential for protein arrays, Biacore and Millennium Pharmaceuticals announced a joint research venture in 2000 to develop an SPR array. Biacore has now developed a prototype SPR array that the company claims can simultaneously assay <50 spots, with no compromise on the high quality of information and high sensitivity that the original four-spot system possessed (FIG. 6a,b). This instrument will allow panels of markers to be studied with increased throughput, in line with the trend in drug discovery towards high information screening. In the future, a larger array could be made when the applications and biological content demand higher throughput.

Graffinity Pharmaceuticals has also developed an SPR-array biosensor — the Plasmon Imager. In contrast to most other SPR instruments, the Plasmon Imager uses wavelength-dependent measurement to detect binding of biomolecules to their respective ligands. Here, a two-dimensional sensor array is imaged onto a spatially resolving detector, and the wavelength of the light that illuminates the sensor array is scanned. So, the resonance spectrum of each single-sensor field or spot of the array is obtained (FIG. 6c). When the buffer solution that serves as a reference is exchanged against the same solution that contains the protein in question, the resonance spectrum is shifted at the position of those sensor fields at which an immobilized compound is bound by the protein. The wavelength shift allows discrimination of different binding strengths of the immobilized ligands. Graffinity Pharmaceuticals claims that parallel measurements of this kind can be done with 1,536 to 9,216 small-molecular-mass compounds immobilized on the sensor surface.

HTS Biosystems uses an alternative approach to SPR, called grating-coupled SPR or GCSPR. In this case, the biosensor chip is made of plastic, and can be moulded to include other features, such as channels,

reaction chambers and fluid coupling ports. By moulding a fine grating onto the surface of a CD-ROM, numerous addressable active sites could be created at very low cost (FIG. 6d). The CD-ROM could then be changed, as in a jukebox, allowing for low-technology automation and sensing. HTS Biosystems claims formats will also include single chips or 96- and 384-well microplate-compatible footprints with multiple high-density arrays.

SRU Biosystems exploits a phenomenon called colorimetric resonant reflection, which uses a surface that reflects a narrow band of wavelengths when illuminated with normal white light. The sensor structure can be produced at low cost in continuous sheets of plastic film and incorporated into various standard-laboratory-assay formats, such as 96-, 384- and 1,536-well microtitre plates, microarray slides and fluid-flow channels. For further information on other instrumentation and multiplexed configurations, the reader is referred to the detailed online TABLE 1 that accompanies this article.

Arrays have allowed a massive improvement in assay throughput with a high level of flexibility in experimental design. Potential applications of multiplexed, label-free screening include: cell-proteome screening; high-throughput target identification; high-throughput screening of arrayed small molecules, arrayed antibodies and arrayed peptides; and, finally — for high-throughput ADME/toxicology — receptor arrays for on-chip drug profiling. In addition, the simultaneous interrogation of multiple reference sites brings with it several important technical advantages. Multiple sites can be used to probe the different levels of signal shifts using repeated standards to improve the quality of the binding data. Sites can be designated as positive and negative controls for biology, chemistry or hardware. These *in situ* controls should reduce the need for more expensive engineering and production steps by normalizing for artefacts such as transducer inhomogeneity, uneven sample introduction and uneven temperature control.

A bright future for optical biosensors

There are an increasing number of commercially available instruments (TABLE 1) that are driving forward the development of novel sensor surfaces, immobilization techniques and attachment chemistries, which together allow virtually any receptor–analyte complex to be screened. A label-free screening system imparts enormous flexibility to the process of assay design and facilitates successful integration with other technologies. Scientists in both academia and industry are using optical biosensors in areas that encompass almost all stages of the drug discovery process. The advent of optical-biosensor arrays will accelerate acceptance of optical biosensors in new areas of drug discovery, for which high information content, rather than ultra-high throughput, is important. The impact of optical biosensors in drug discovery will therefore continue to grow over the next decade.

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This paper shows that interaction affinities, kinetics and thermodynamics can all be obtained using an optical biosensor.

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