

Direct Optical Detection of Protein–Ligand Interactions

Frank Gesellchen, Bastian Zimmermann, and Friedrich W. Herberg

Summary

Direct optical detection provides an excellent means to investigate interactions of molecules in biological systems. The dynamic equilibria inherent to these systems can be described in greater detail by recording the kinetics of a biomolecular interaction. Optical biosensors allow direct detection of interaction patterns without the need for labeling. An overview covering several commercially available biosensors is given, with a focus on instruments based on surface plasmon resonance (SPR) and reflectometric interference spectroscopy (RIFS). Potential assay formats and experimental design, appropriate controls, and calibration procedures, especially when handling low molecular weight substances, are discussed. The single steps of an interaction analysis combined with practical tips for evaluation, data processing, and interpretation of kinetic data are described in detail. In a practical example, a step-by-step procedure for the analysis of a low molecular weight compound interaction with serum protein, determined on a commercial SPR sensor, is presented.

Key Words: Optical biosensors; surface plasmon resonance; reflectometric interference spectroscopy; biomolecular interaction analysis; kinetics; low molecular weight ligands.

1. Introduction

A functional description of biomolecules has to extend beyond a solely static description of the protein content within a cell, a cellular compartment, or a tissue. A detailed kinetic description of the interaction patterns has to be added, because these molecules are involved in a dynamic equilibrium. Several methods employing different physical principles have been adopted to determine the binding of one biomolecule to another or to monitor complex formation. This can be done by combining a detector, very often an optical mass detector,

with a microfluidics system and monitoring the interaction of a component immobilized to a solid phase, in the following termed **ligand**, and an **analyte** in flow phase (see **Fig. 1**). Biomolecular interaction analysis (BIA) describes highly accurately relevant binding events between compounds under physiological conditions, thereby providing detailed kinetic data of the association and dissociation of binding partners. Being a part of functional genomics, BIA is also implemented in drug development and quantifies the interactions between small molecules, proteins (e.g., receptors, enzymes, antibodies), peptides, nucleotides, carbohydrates, and other biomolecules. In combination with systematic molecular and cellular analyses of proteins, BIA assigns function to arbitrary listings of gene products.

A typical interaction analysis is based on three steps: 1) coupling of the ligand, 2) interaction analysis, and 3) regeneration.

1. The ligand has to be immobilized in an appropriate manner maintaining the biological activity as well as providing a stable binding to the solid support (see **Subheadings 1.3.1. and 3.1.** for details).
2. Once the ligand surface displays a stable baseline, the analyte is added using a well controlled flow system. This allows monitoring association and dissociation phases separately and plotting them in form of a *sensorgram* (see **Fig. 2**). Using serial dilutions of analyte, the association rate constant (k_a) and dissociation rate constant (k_d) are extracted with an appropriate software applying pseudo-first order kinetics. With the known concentration of the analyte, apparent equilibrium binding constants (K_D or K_A) can be calculated. Besides the kinetic constants, EC_{50} values for competitors can be determined by solution competition or surface competition as described later. Special care has to be taken to subtract nonspecific or unspecific binding events. In a multichannel system, this can be performed by subtracting the binding on a reference surface. A reference surface either lacks the specific ligand or, preferably, is modified with an appropriate negative control.
3. After the interaction has taken place, bound analyte has to be removed completely from the ligand surface to perform another interaction analysis. However, except in the case of transient interactions, baseline level is seldom reached in an acceptable time frame. Therefore, a procedure referred to as regeneration has to be performed, removing bound analyte with appropriate agents without destroying the biological activity of the immobilized ligand (see **Fig. 2**). Appropriate conditions should be optimized for individual interaction partners, common methods include for example treatment with glycine at acidic pH in the case of antibody mediated interactions. Optimally, a biospecific regeneration procedure can be used, as shown in **Fig. 2** for the interaction between the catalytic (C-) and the regulatory (R-) subunit of cAMP dependent protein kinase (PKA). Dissociation of the regulatory subunit from the catalytic subunit is initiated by cAMP, therefore a C-subunit surface can be regenerated by an injection of this physiological effector. An overview of possible regeneration conditions for differently immo-

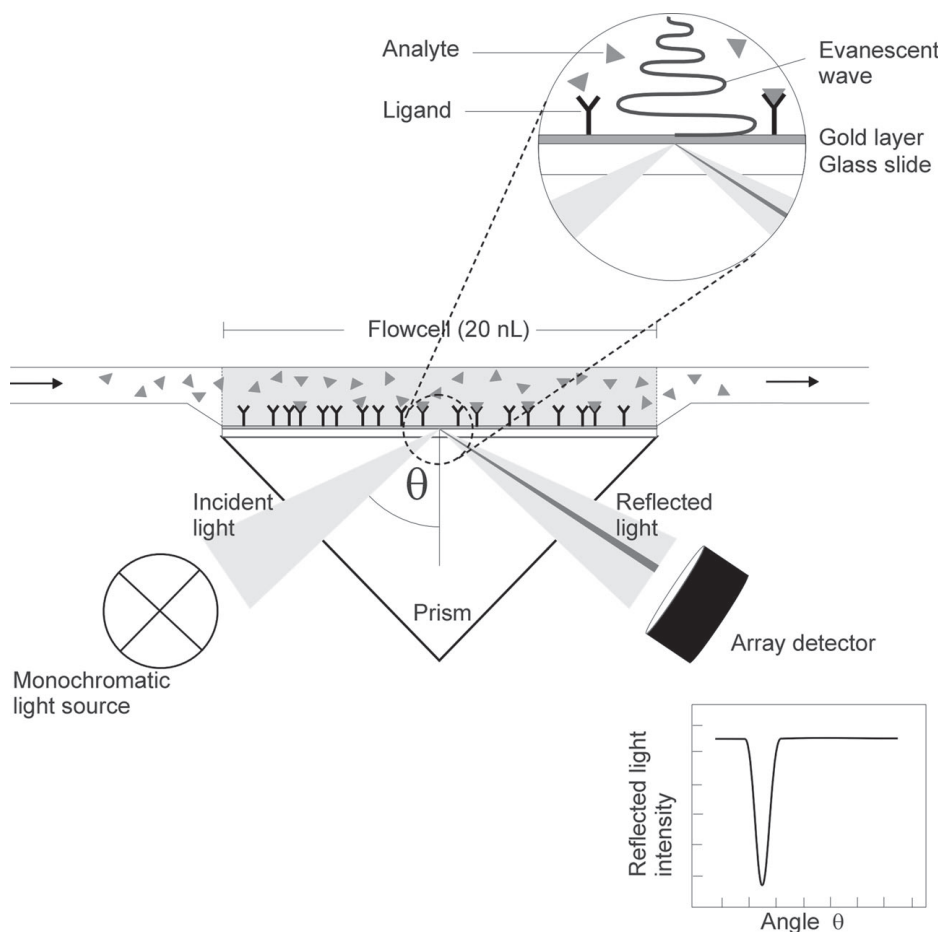


Fig. 1. Detection principle of a SPR-biosensor (*see Subheading 1.1.1.* for details). Monochromatic light focused in a wedge on a metal-coated interface between two media with a high refractive index (glass) and a low refractive index (solution), respectively, is totally internally reflected. At a specific angle (the so-called resonance angle θ) the incident light is coupled into the plasmons of the metal layer that results in emission of an evanescent wave into the lower refractive index medium (*see inset*). The ensuing drop in light intensity appears as a shadow in the reflected light wedge, which is monitored on the position-sensitive diode array detector. The resonance angle is dependent on the refractive index of the solution close to the surface layer and hence on the amount of analyte bound to the immobilized ligand (*see text* for details).

bilized ligands is given by Herberg and Zimmermann (*1*). Biacore AB is compiling a database of immobilization and regeneration procedures on their website (<http://www.biacore.com>) that should prove very helpful to the user.

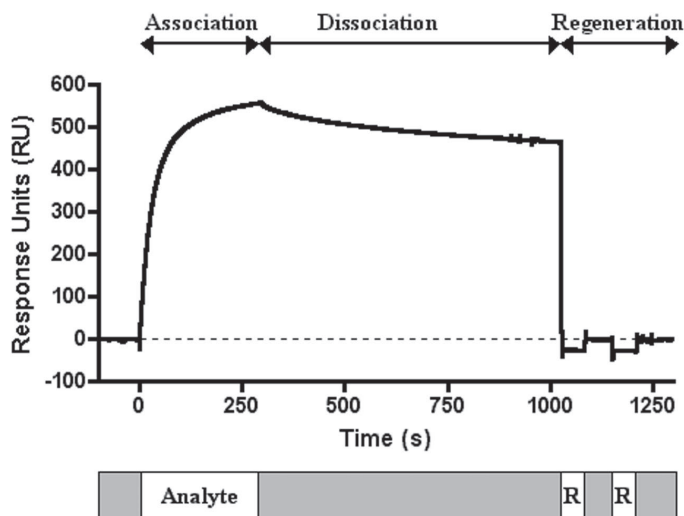


Fig. 2. Typical sensorgram of an interaction analysis. The figure shows the interaction of the regulatory subunit with the catalytic subunit of cAMP dependent protein kinase immobilized by amine coupling. During injection of the analyte (as indicated below the x -axis), binding to the ligand is reflected by an increasing signal throughout the association phase. Dissociation starts when the analyte is omitted from the running buffer. In order to return to baseline level (indicated by dashed line) for a new cycle of injections, the surface is regenerated with short pulses of regeneration solution (R, in this case 0.1 mM cAMP, 2.5 mM EDTA, *see text for details*).

1.1. Instrumentation

Optical detection principles for monitoring of biomolecular interactions have been implemented into various commercial biosensors. Most optical biosensors consist of three main components: a detector based on different physical/optical principles, a sample delivery system (microfluidics), and the sensor surface where one of the interaction partners is immobilized either covalently or noncovalently. In the following, surface plasmon resonance (SPR)-based detectors—with special emphasis on the Biacore technology—will be discussed in more detail. Additionally, reflectometric interference spectroscopy (RIfS) is presented as a very promising technology in biomolecular interaction analysis, followed by a brief introduction of commercially available biosensors.

1.1.1. Surface Plasmon Resonance-Detectors

Traditionally, SPR has been used to determine binding constants for macromolecular interactions, owing to the fact that SPR sensors can be utilized as optical mass detectors. SPR occurs when light illuminates thin conducting films

(gold in the case of Biacore instruments) under specific conditions. The resonance is a result of the interaction between electromagnetic vectors in the incident light and free electron clouds, called plasmons, in the conductor. SPR arises as a result of a resonant coupling between the incident light energy and the surface plasmons in the conducting film at a specific angle of incident light. Absorption of the light energy results in the emission of an evanescent wave into the low refractive index medium, which causes a characteristic drop in the reflected light intensity at that specific angle (*see Fig. 1*).

The resonance angle θ is sensitive to a number of factors, including the wavelength of the incident light, the nature and thickness of the conducting film and the temperature. Most importantly for this technology, the angle depends on the refractive index of the medium opposite of the incident light. When all other factors are kept constant, the resonance angle is a direct measure of the refractive index of the medium. Only the angle at which SPR occurs is altered and detected with the diode array detector; the intensity of the shadow in the reflected light is unchanged. Binding events cause changes in the refractive index at the surface layer, which are detected as changes in the SPR signal. In general, the refractive index change for a given change in mass concentration at the surface layer of a sensor chip is practically the same for all proteins and peptides (2), thereby providing a sensitive real-time mass detector. However, for glycoproteins, lipids, and nucleic acids this refractive index change is slightly different. Suitable calibration procedures using standard substances still allow determination of correct values for binding, plotted as response units (RU). For a general purpose sensor chip, the CM5 chip (Biacore AB, Sweden), 1000 RU correspond to 1 ng protein/.mm² sensor surface (2) and generate a shift of 0.1° in the resonance angle θ .

The technology has been implemented into several instruments already available or under development from Biacore AB. For basic research, Biacore X (two flow cells), Biacore 2000, and 3000 (four flow cells), and for higher throughput, Biacore TAS (eight flow cells), were developed, and an array instrument for high throughput is under construction. The Biacore S51 instrument with lower sample consumption and higher resolution is aimed specifically at drug screens.

1.1.2. Reflectometric Interference Spectroscopy

Reflectometric interference spectroscopy (RIfS) is a BIA technology that has—although already in use for several years—only recently been implemented into a commercially available biosensor, the BIAffinity instrument just introduced by Analytik Jena AG (Jena, Germany). So far, however, almost all research data based on RIfS have been collected using custom built detectors in academic research laboratories as described below.

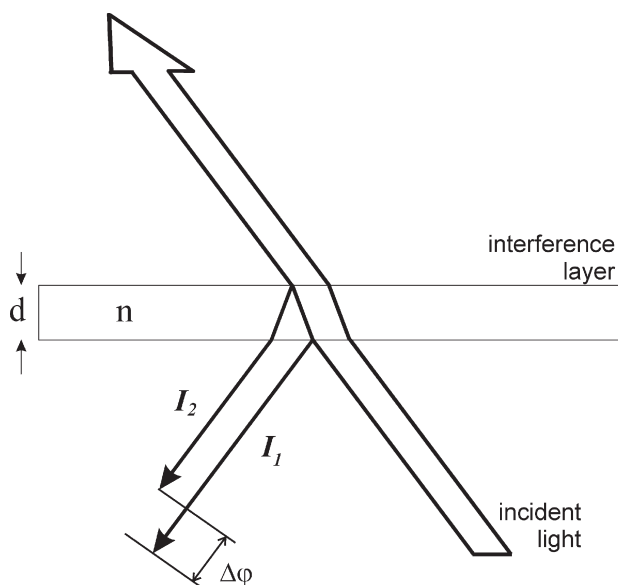


Fig. 3. Detection principle of RIfS. Incident white light is partially reflected at each surface of an interference layer with a refractive index n and a physical thickness d . Reflected light beams of intensities I_1 and I_2 travel different optical path lengths, resulting in a phase difference $\Delta\phi$, which produces a distinct interference pattern of alternating minima and maxima. Binding of biological material at the surface increases the optical thickness ($n \times d$) of the interference layer, leading to a corresponding shift in the interference pattern. (Adapted from **ref. 3**.)

RIfS exploits an optical phenomenon occurring at thin transparent films: a light beam passing a weakly reflecting thin film will be reflected in part at each of the interfaces (*see Fig. 3*). As the reflected light beams travel different optical pathlengths, a phase difference is introduced, resulting in a distinct interference pattern of alternating minima and maxima. This phenomenon is dependent on the angle of incidence and the wavelength of the incident light as well as the physical thickness of the film and its refractive index. Binding of biological material to the surface causes a change in the optical thickness of the film. The increase in optical thickness results in a shift of the reflectance pattern which can be monitored with high resolution in real-time with a simultaneous diode array spectrophotometer.

For biomolecular interaction analysis, glass chips with an interference layer of 500 nm SiO_2 deposited on top of 10 nm Ta_2O_5 for reflection enhancement are used. To reduce nonspecific binding the interference layer must be modi-

fied. This is done by silanization with aminobutyl-dimethylmethoxysilane and covalent coupling of hydrophilic polymers (dextran, polyethyleneglycol) (3). Binding curves can be recorded using a simple setup consisting of a 20 W tungsten halogen lamp and a diode array spectrophotometer connected by a 2:1 fiber-optic coupler.

The general applicability of RIfS for monitoring interactions between biomolecules and low molecular weight ligands has been demonstrated by several applications including the characterization of the streptavidin-biotin interaction (4), antibodies binding to a hapten (3), as well as the interaction between DNA and DNA-binding compounds (5). The technique is also well suited for parallelization, and has been used for high-throughput screens of thrombin inhibitors (6), for screening of a combinatorial triazine library with different antibodies (7), and for epitope mapping (8).

The applications described above suggest sensitivity and detection limits for RIfS in the same order of magnitude as for other commonly employed optical biosensors (9). The RIfS technology provides a simple and robust alternative to the Biacore SPR sensors. While not quite reaching the high sensitivity of the Biacore instruments, it is nevertheless capable of generating reproducible interaction data over a high dynamic range despite the lack of a sophisticated microfluidics system (9). An advantage of RIfS is that the detection principle itself is not temperature sensitive, and thus does not require the expensive temperature control systems crucial for SPR-based sensors. Finally, the capability of parallelization and high-throughput screenings make RIfS an attractive technology in the field of optical biosensors.

1.1.3. Other Optical-Based Technologies

Aside from the Biacore instruments, the SPR technology has also been implemented into other commercially available instruments, such as the Instrument of Biomolecular Interaction Sensing (10) (IBIS, Windsor Scientific, Slough, UK), which utilizes a cuvet based setup, or the Spreeta device from Texas Instruments (Dallas, TX), a miniaturized portable SPR platform (11). Another related physical principle used to monitor biomolecular interactions is the resonant mirror (12) that is implemented in the IAsys system (ThermoFinnigan, San Jose, CA). Aside from the differences in the detection principle, the instrument uses a cuvette based sample delivery system.

Another application of SPR technology takes advantage of the evanescent waves generated in fiber-optics waveguides when the propagated light beam is totally internally reflected at the wall of the fiber. A fiber-optic sensor specifically aimed at quantifying protein and small molecule interactions, the LunaScan device, has been patented by Luna Analytics (Blacksburg, VA). This biosensor uses long period gratings (LPG) inside an optical fiber to scatter

light from the optical fiber at a predetermined wavelength that is dependent on the grating period. The light scatter in turn is dependent on the refractive index of the fiber and its surrounding environment. Upon adsorption of target analytes to the surface coating, the resulting refractive index change causes a spectral shift in the wavelength of the scattered light that is proportional to the mass of analyte bound.

The FLEX CHIP Kinetic Analysis System by HTS Biosystems (Hopkinton, MA) employs grating coupled SPR, where the incident light is coupled into the surface plasmon via an optical grating. An instrument based on the same principle, the Applied Biosystems 8500 Affinity Chip Analyzer is also aimed at high throughput analyses. According to the manufacturer, the instrument is capable of measuring interactions with K_D from the pM to the μM range.

A novel biosensor developed by SRU Biosystems (Woburn, MA) utilizes a colorimetric resonant diffractive grating surface as surface binding platform (13). The grating is designed in such a way that, when illuminated with white light, it reflects only light of a single wavelength. Attachment of molecules to the surface shifts the reflected light wavelength due to the change of the optical path of light coupled into the grating. This method is capable of resolving changes of 0.1 nm thickness of protein binding on the surface and is well suited for miniaturization and parallelization (13). For other recent developments in the field of optical biosensors the reader is referred to a comprehensive review by Baird and Myszka (14).

1.2. Applications

1.2.1. Basic Considerations

Based on sales and on the amount of scientific literature published, SPR-based devices, for example the Biacore instruments, are the most commonly used commercially available biosensors (15). Typical applications include analysis of protein–protein or protein–DNA interactions, characterization of antibodies (epitope mapping), elucidation of the influence of post-translational modifications on interaction kinetics, but also the investigation of macromolecular complexes up to supramolecular compounds like viruses, microorganisms, or entire cells. On the opposite end of the scope stands BIA of low molecular weight compounds with proteins, which is of special interest in drug research (16). For hit validation and optimization of lead compounds a detailed kinetic characterization of pharmaceutical substances is required. A potential limitation of SPR sensors lies in the detection principle: a mass change on the sensor surface is transduced into a proportional optical signal, i.e., a small increase in mass on the surface results in an accordingly small signal. Therefore, it appears favorable to immobilize the low molecular weight ligand on the sensor surface and use the larger interaction partner as the analyte in the soluble

phase. Immobilization of small ligands, however, often requires their previous derivatization and care must be taken to determine the effect of the modification on ligand functionality. Another caveat of this approach is that high-density surfaces of ligand are prone to mass transfer limitations (*see Note 2*), whereas low density surfaces—which are suitable for kinetic analyses—are difficult to adjust with small ligands, because the immobilization process cannot be observed easily as a result of the low response. Another problem is a reduction in degrees of freedom inherent to the immobilization process that can severely affect the rate constants. On the other hand, immobilization procedures for large molecules (i.e., proteins) are well established (*1*) and recent advances in instrumentation (SPR based biosensors like the Biacore 3000 and S51), in the analysis software, and suitable calibration procedures allow direct optical detection of small molecule binding.

1.2.2. Ligand Interaction in the Indirect Assay Format

Alternatively, binding of low molecular weight substances can be assessed by solution or surface competition assays (*see Fig. 4*) comparable to the procedures already in use for immunoassays such as radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) (*17*). In solution competition experiments, the competitor molecule interferes with binding of the analyte to the immobilized ligand, whereas in surface competition the molecule of interest competes with the analyte for the same binding site (*see Fig. 4*).

Shortly after the introduction of the first SPR biosensor by Biacore in 1990, Karlsson (*18*) described a competitive kinetics approach for characterization of low molecular weight ligands, using the binding of HIV p24-derived peptides (competitor 1) vs the intact antigen (competitor 2) to a monoclonal antibody as a model system. This assay format continues to be a valuable tool, as it has been used to determine levels of small metabolites like morphine-3-glucuronide, the main metabolite of heroine and morphine (*19*), or deoxynivalenol, a highly toxic fungal metabolite, that may contaminate food and animal feed (*20*).

1.2.3. Ligand Interaction in the Direct Assay Format

However, in recent years researchers have increasingly employed the direct binding assay for BIA analysis of low molecular weight ligands (*15*). The validity of interaction data acquired with SPR has been ascertained by comparison with stopped-flow fluorescence and isothermal titration calorimetry measurements (*21,22*). Compared to other approaches, SPR biosensors have the advantage of providing a relatively robust readout, a simple assay format, and low sample consumption. The binding event can be monitored directly without the need for labeling of one or both of the interaction partners. Further-

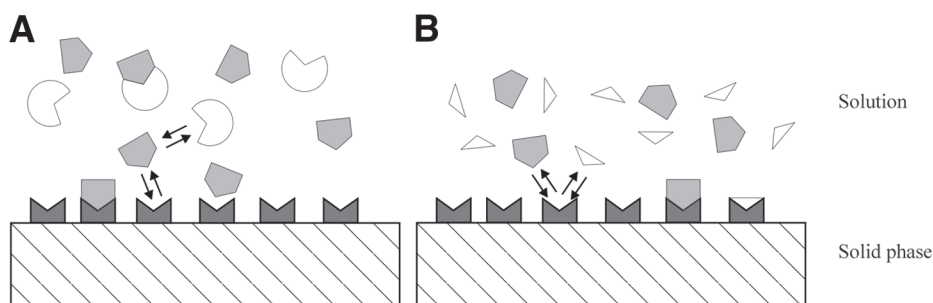


Fig. 4. Competition experiments. Schematic setup of competition experiments. By varying the concentration of the competitor, EC_{50} values can be deduced. (A) *Solution competition*. Soluble receptor molecules (white) bind to the analyte in solution (light gray), thereby competing with analyte binding to the ligand immobilized to the surface (dark gray). Only binding of free analyte to the ligand is detected. (B) *Surface competition*. A competitor molecule (white) competes with the analyte (light gray) for the same binding site on the ligand (dark gray). For use of this assay with biosensors based on SPR, a significant difference in molecular weight between analyte and competitor is required.

more, higher information content is given, because this technology allows the user to measure association and dissociation rate constants separately. One can determine kinetic as well as equilibrium binding data in a single experiment. Additionally, even thermodynamic data of the respective binding event can be extracted once a series of experiments is performed at different temperatures (23). Moreover, the whole process can be automated and used for screening of compounds in a medium throughput format.

The interactions of low molecular weight substances with target molecules have been investigated by several groups using Biacore technology. Among many similar studies the usefulness of SPR-based biosensors for drug screens has been demonstrated by describing binding kinetics and affinities of 58 different inhibitors to HIV-1 proteinase (24), and by a screen of 170 compounds for binding to immobilized thrombin (25).

On the very extreme end of the spectrum Gestwicki and co-workers (26) were able to detect binding of maltose to maltose-binding protein (MBP) and calcium ions to tissue transglutaminase (tTG), respectively, using a Biacore instrument. The resulting SPR signal could not be attributed to the actual binding event, because it was either negative (in the case of maltose-MBP interaction) or too high to be explained by binding of the small analyte (in the case of Ca^{2+} binding to tTG). According to the authors, a possible explanation could

be a conformational change of the receptor protein upon analyte binding, which in turn may induce refractive index changes close to the matrix.

Aside from protein interaction studies, the binding of small molecules to nucleic acids is an important issue in the biomedical field. The coupling of DNA/RNA to the sensor surface is simplified by a previous biotinylation of the nucleic acid followed by immobilization on a streptavidin coated sensor chip (see **Note 1**).

Using biotinylated DNA-hairpin oligomers the mode of action and sequence specificity of the DNA binding antitumor antibiotic AT2433-B1 was successfully identified with SPR analyses complementing DNase footprinting experiments (27). This approach has been used in several similar studies with antitumor drugs binding to DNA. Accordingly, the same strategy is also applicable to RNA, as exemplified by several studies of therapeutics binding to RNA molecules (28–30).

For interaction screens in the direct assay format it is crucial that the immobilized ligand retains its biological activity during the entire set of experiments, which should be checked routinely by injection of a reference analyte. Another issue generally relevant for the analysis of small analytes is unspecific binding either to the chip matrix or to the immobilized protein. The extent of such unspecific binding should be assessed and corrected for by injection over appropriate reference surfaces. Likewise, the influence of solvents such as DMSO can be addressed by calibration on a reference surface. A detailed description for the characterization of the binding behavior of a low molecular weight compound (here: warfarin) with immobilized serum albumins from different species is given under **Subheading 3.5.** of this article.

1.3. Interaction Analysis

An essential feature of direct optical detection systems is the immobilization of the ligand molecule in order to detect the binding of a soluble analyte. Most optical biosensors are based on glass and/or metal surfaces that have to be derivatized to generate a biocompatible environment. Carboxymethylated dextrans with a low degree of crosslinking have been proven to be excellent for ligand coupling as they allow to generate high surface densities and display low unspecific binding. At the same time, a hydrophilic matrix for the biological interaction close to the sensor surface is provided.

1.3.1. Immobilization

An accurate kinetic analysis can only be performed, if the ligand molecule is immobilized in a biologically active conformation. Steric hindrance caused by the immobilization strategy can be a serious problem, either prohibiting, reducing, or modulating the respective binding. Therefore, a suitable experi-

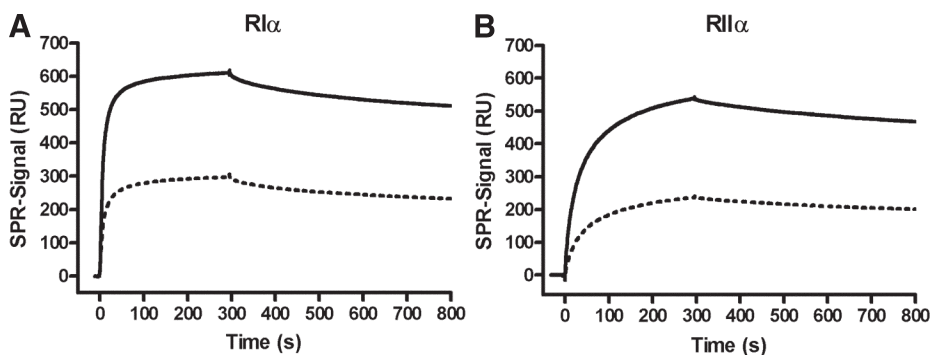


Fig. 5. Influence of MgATP on immobilization of the C-subunit of PKA. Recombinant C-subunit was immobilized on a CM-dextran surface via primary amines. 730 RU and 870 RU of C-subunit in 10 mM acetate buffer pH 6.0 were coupled, in the absence and presence of MgATP (1 mM ATP, 2 mM MgCl_2), respectively. A reference surface was treated accordingly without injection of a ligand. After immobilization, 400 nM recombinant RI α (A) or RII α subunits (B) were injected in running buffer (20 mM MOPS pH 7, 150 mM NaCl, 2 mM MgCl_2 , 1 mM ATP, 0.005% Tween-20) and the association monitored for 5 min before the dissociation phase was started by injection of running buffer. No unspecific binding was monitored on the reference surface. After 10 min of dissociation, the surface was regenerated to baseline level by injection of 0.1 mM cAMP, 2.5 mM EDTA (not shown). The binding stoichiometry to both inhibitors was increased by a factor of two when adding MgATP (solid lines) during the immobilization compared to C-subunit immobilized without MgATP (dashed lines).

mental setup has to be generated to check for biological activity of the immobilized ligand.

This is exemplified when looking at interaction partners of the catalytic subunit of cAMP dependent protein kinase. If the catalytic subunit is immobilized to the sensor surface using primary amines, coupling can also occur via Lys72, a residue that is essential for the correct conformation of the catalytic site, which in turn is a prerequisite for efficient binding of physiological kinase inhibitors. To avoid coupling via Lys72, MgATP is added during the immobilization procedure to occupy the active site cleft of this protein kinase. When this cosubstrate of the enzyme is bound, the critical lysine residue is protected from modification. If Lys72 is not protected during immobilization, binding of physiological inhibitors is negatively influenced, reflected in a decreased binding stoichiometry. **Figure 5** shows the interaction of two different physiological inhibitors of the catalytic subunit, the regulatory subunit type I and type II.

Although those inhibitors occupy distinctly different surface areas of the catalytic subunit, both require interaction with the active site for high-affinity binding (31). The binding stoichiometry in the presence of the protective cofactor MgATP is increased by approx 100% (from 33 to 66% in the case of the type I regulatory subunit and from 33 to 62% in the case of RII subunit, assuming binding in a 1:1 molar ratio). Interestingly, the binding pattern, as indicated by the shape of the curves, does not differ significantly between catalytic subunit immobilized in the presence and absence of MgATP, respectively, suggesting that only catalytic subunit immobilized in an active conformation participates in the binding. This is also reflected in the apparent association and dissociation rate constants calculated from serial dilutions of the regulatory subunit (data not shown).

1.3.2. Detection of Small Molecule Ligand Interactions

A wide range of biological interactions can be analyzed using SPR biosensors. For reasons discussed earlier, direct optical detection of low molecular weight compounds is a challenge. As an example, in the early phase of the drug development process the determination of adsorption, distribution, metabolism, and excretion (ADME) parameters has become very important (16). In this context, the interaction of low molecular weight compounds to serum proteins such as human serum albumin or alpha acid glycoprotein is investigated. High-affinity binding to serum proteins significantly changes the physiologically active concentration of a compound and thus reduces its bioavailability, but also prolongs its duration of action because of a slower release from the bound state. Although a wide range of methods such as equilibrium dialysis, ultracentrifugation, spectroscopic, and chromatographic approaches are available to monitor binding of small analytes to serum proteins, direct optical detection methods have advantages as a result of low sample consumption, high accuracy, and reproducibility and do not require labeling. Furthermore, the potential for parallelization and automation makes this technique suitable for screening assays with increased throughput. The experiment depicted in **Figs. 6** and **7**, shows the interaction of warfarin, a low molecular weight compound (molecular weight 308.3 Da), with serum albumins of human (HSA), bovine (BSA), and murine (MSA) origin, examined in parallel on a Biacore instrument. This allows a direct comparison of serum albumin binding levels and a prediction of bioavailability in different biological systems such as cell culture, transgenic animals, or in humans. Interestingly, significantly different binding patterns could be observed for the three mammalian serum albumins investigated, yielding K_D -values of 3.6 μM , 7.8 μM , and 49.8 μM for HSA, BSA, and MSA, respectively (*see Fig. 7*). The binding data for HSA are in excellent agreement with results provided by Rich et al. (32).

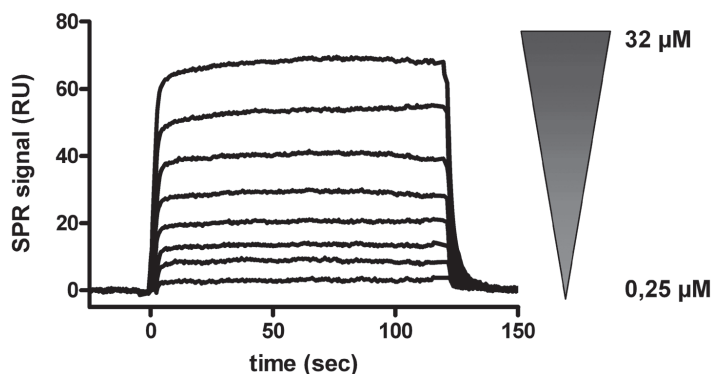


Fig. 6. Reference subtracted binding curves of warfarin to human serum albumin (HSA, 20000 RU immobilized). Warfarin in running buffer containing 3% DMSO was injected in concentrations ranging from 250 nM to 32 μ M. A representative set of data is presented.

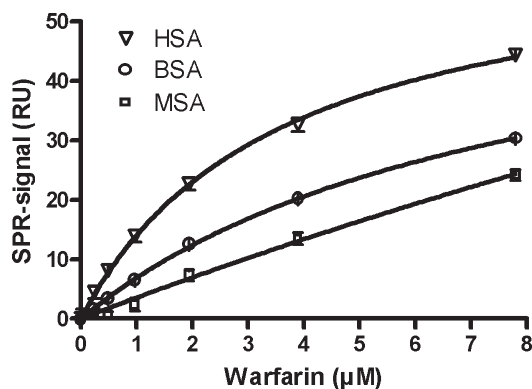


Fig. 7. Equilibrium binding analysis of warfarin to three different serum albumins of HSA, bovine BSA, and MSA origin. Here, the reference subtracted and solvent corrected steady-state binding levels are plotted against the respective warfarin concentrations. K_D -values for each data set are extracted by nonlinear regression plots of the saturation curves.

In principle, serum protein binding assays using SPR biosensors are relatively simple to perform and can be used in routine analysis. However, some experimental details are crucial for a successful realization of the experiment and will be described in the following. Serum albumin proteins are coupled to high-surface densities using standard amine coupling (*see Subheading 3.5.1.*)

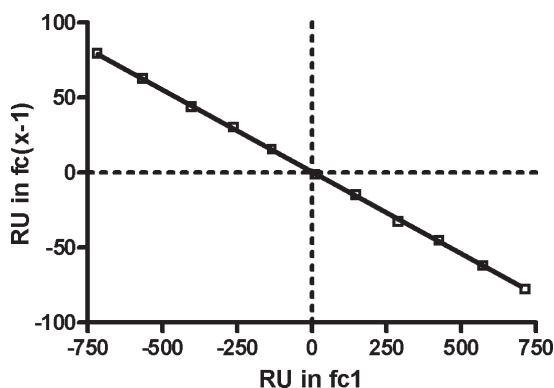


Fig. 8. DMSO calibration curve for concentration series of solvent in running buffer. DMSO in running buffer, ranging in concentrations 0.5% above and below (in 0.1% increments) the DMSO concentration in running buffer, was injected over the specific (fcx) as well as the reference surface (fc1). The signal on the reference cell (fc1, x-axis) is plotted against the reference subtracted signal on the specific surface (fc[x-1], y-axis). Correction factors are calculated by inserting the signal on the reference surface (RU in fc1) as x-value into the equation obtained from the linear regression of the calibration curve. The resulting y-value (RU in fc[x-1]) is the corresponding correction factor. For further details *see Subheading 3.5.3*.

to obtain an acceptable signal-to-noise ratio. Common problems related to high-surface densities like mass transfer limitations (*see Note 2*) do not apply to the kinetics of small molecule analytes because small molecules have favorable diffusion properties. However, as a result of their limited solubility in aqueous solutions, most low molecular weight compounds have to be dissolved in buffers containing organic solvents like dimethyl sulfoxide (DMSO). DMSO itself has a high refractive index and small differences in concentrations induce large increases in signal that have to be subtracted. Furthermore, the refractive index changes might be slightly different for the reference and the specific surfaces demanding a sophisticated calibration procedure (*see Subheading 3.5.2*). The resulting calibration curve enables the calculation of correction factors for the specific samples (*see Fig. 8*). Once the sample is diluted, consider to match the DMSO concentration exactly to the running buffer because the high refractive index changes induced by varying DMSO concentrations will increase the correction factors and make the assay less sensitive. It is essential to check if the compound is still soluble after dilution. At this step it is also very important to avoid evaporation because this will significantly change the buffer composition and the concentration of the compound.

Most low molecular weight compound interactions with serum albumins display fast on- and off-rates, hence it is sufficient to inject each sample for 1 min and report the equilibrium binding response in the middle of the injection phase. A sufficient number of blank injections should be performed between samples in order to prevent carry-over. As a result of the fast off-rates a regeneration of the serum albumin surface is usually not necessary. The reference subtracted and solvent corrected equilibrium binding responses are then plotted against the compound concentration, and the K_D -values for each data set can be calculated by nonlinear regression (*see Subheading 3.5.* for details). Still, interpretation of the data is not always trivial because HSA is known to have several binding sites for small ligands with varying affinities. Therefore, two or more binding sites for one ligand might be observed and reflected in the binding kinetics. For data evaluation it is important to select an adequate range of concentrations because ligand binding to multiple sites causes large variances in the calculated K_D -values depending on the selected concentration range.

1.3.3. Interpretation of Kinetic Data

Several software packages based on linear or nonlinear analysis are available to analyze high-resolution kinetic data (*see Subheading 3.4.*). However, library screens—either of expression libraries or chemically generated libraries—produce a vast number of interaction data that can be difficult to interpret with available software. Software tools have to be developed to classify and visualize bulk kinetic data automatically and to perform basic kinetic evaluations. KineticXpert by Microdiscovery GmbH (Berlin, Germany) is a software tool under development performing bulk analyses and evaluation of interaction data and facilitating data management (<http://www.microdiscovery.com>).

Interpretation of kinetic data can be helpful in elucidating more complex biological mechanisms. Yaqub et al. (33) investigated the domain interaction of C-terminal src kinase (Csk), a member of the src kinase family using surface plasmon resonance. After immobilization of the Csk kinase domain using amine coupling, the immobilized protein was phosphorylated by another kinase (PKA) on the chip, and thereby modulated in its binding behavior. This is reflected in a change in the binding kinetics of an isolated SH3 domain run over the chip before and after phosphorylation (33). Evaluation of the interaction patterns after phosphorylation demonstrated that the kinetics obtained were not compatible with a 1:1 binding model. Careful examination of several different binding models (*see Fig. 9* and legend) led to the conclusion that the immobilized Csk kinase domain had not been phosphorylated completely, resulting in a heterogeneous ligand surface. When the soluble SH3 domain was injected, a slow phase for the unphosphorylated species superimposed a fast phase for the modified, phosphorylated Csk (33). Comparing several models

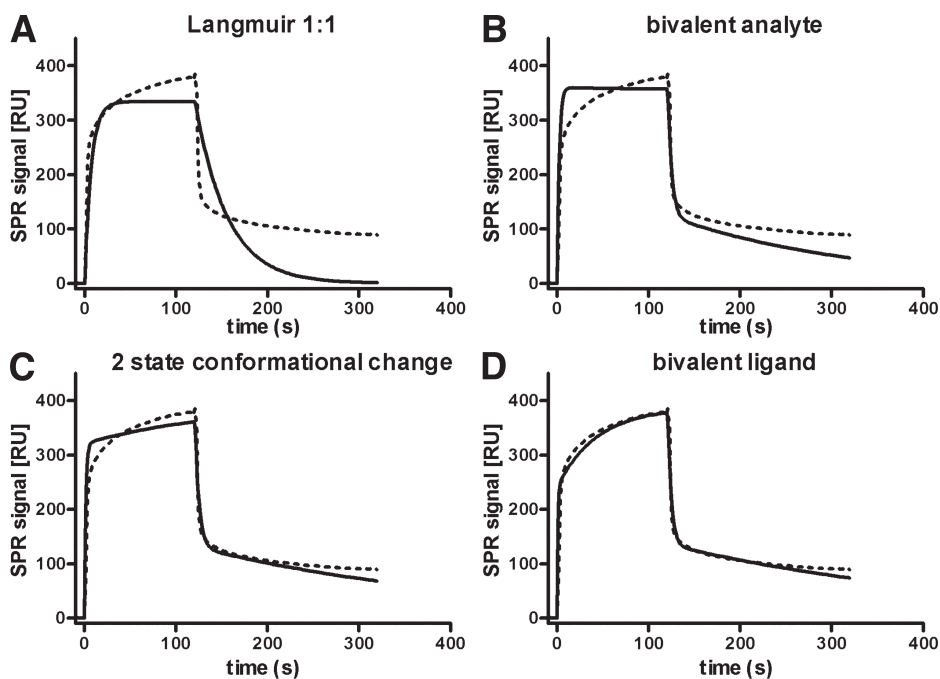


Fig. 9. Interpretation of biological binding data using different kinetic models. $34\ \mu\text{M}$ of an isolated SH3 domain were injected over a surface with immobilized Csk kinase domain which had been on-chip phosphorylated. Panels **A–D** show the respective sensorgram (dashed line) superimposed with fits from different interaction models (solid lines), generated with the BIAevaluation software (v3.2, Biacore AB). Neither a 1:1 binding model (**A**), a bivalent analyte model (**B**) or a two-state conformational change (**C**) yield adequate fitting results. Only the bivalent ligand model (**D**) gives a near-perfect fit with the interaction data, indicating that as a result of incomplete on-chip phosphorylation two distinctly different kinetics were superimposed.

of interaction, the bivalent ligand model yielded by far the best fit with the experimental data (*see* **Fig. 9**, panel **D**). These data demonstrate that biological function can be maintained and modulated even if a molecule is immobilized to a solid phase. Furthermore, these data show that appropriate models describing the biomolecular interaction of choice can generate significant information on the nature of the biological interaction.

2. Materials

The chemicals listed are intended for use with the Biacore system, however, they can easily be adopted for other biosensor devices where one interaction partner has to be immobilized.

2.1. Coupling

Basically, two different covalent coupling chemistries are commonly used: coupling via primary amines using NHS/EDC or coupling via thiols, either by surface or ligand thiol coupling.

2.1.1. Amine Coupling

When performing an *amine coupling* a ligand with a primary amine function (for example a free N-terminus or a lysine residue) is needed.

1. Appropriate immobilization buffer with low ionic strength ranging from pH 3.0 to 6.0, this buffer should contain no primary amines (i.e., do not use Tris-buffers, instead prepare acetate, phosphate or MES (2-(*N*-Morpholinoethanesulfonic acid)) buffer in the intended pH range).
2. 100 mM *N*-hydroxysuccinimide (NHS).
3. 400 mM *N*-ethyl-*N'*-(dimethylaminopropyl)-carbodiimide (EDC).
4. 1 M Ethanolamine hydrochloride, pH 8.5.
5. Ligand solution: 1–100 µg/mL ligand in an appropriate immobilization buffer.

2.1.2. Thiol Coupling

Thiol coupling provides an alternative to amine coupling, and is recommended for ligands where amine coupling cannot be used or yields unsatisfactory results, e.g., for acidic proteins or peptides. Generally, thiol coupling is performed in two different approaches: coupling via intrinsic thiol groups of the ligand (e.g., cysteines), or coupling via thiol groups introduced into carboxyl or amino groups of the ligand (e.g., engineered cysteine residues).

2.1.2.1. INTRINSIC LIGAND THIOL COUPLING

1. Ligand solution: 10–200 µg/mL ligand in an appropriate immobilization buffer.
2. 80 mM 2-(2-pyridinyldithio)-ethaneamine hydrochloride (PDEA) in 0.1 M borate buffer pH 8.5, freshly prepared.
3. 50 mM L-cysteine, 1 M NaCl in 0.1 M formate buffer, pH 4.3 (cysteine/NaCl), freshly prepared.

2.1.2.2. SURFACE THIOL COUPLING

1. Ligand solution: 1 mg/mL in 0.1 M MES buffer, pH 5.0.
2. Fast desalting column (NAP10 column [Amersham Biosciences] or equivalent).
3. 40 mM Cystamine dihydrochloride in 0.1 M borate buffer, pH 8.5.
4. 0.1 M DTT (dithiothreitol) or DTE (1,4-dithioerythritol) in 0.1 M borate buffer, pH 8.5.
5. 20 mM PDEA, 1 M NaCl in 0.1 M sodium formate buffer, pH 4.3 (PDEA / NaCl), freshly prepared.

2.1.3. Noncovalent Coupling

Noncovalent coupling is performed using fusion tags, by employing biotinylated components, or by generating lipid-containing sensor surfaces. Fusion pro-

teins are captured via site-specific antibodies against the fusion tag, for example, anti GST or anti poly-His antibodies. For poly-His fusion proteins a patented Ni-NTA sensor chip can be used (*see Note 1*) and the following materials are needed:

1. Running buffer: 10 mM HEPES (*N*-[2-Hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid]), pH 7.4, 150 mM NaCl, 50 μ M EDTA (ethylenediaminetetraacetic acid), 0.005% Tween-20, filtered and degassed.
2. Nickel solution: 500 μ M NiCl₂ in running buffer.
3. Ligand solution (do not use additional EDTA or bivalent metal ions in the buffer; nonspecific binding can be prevented by varying ionic strength and pH; addition of 10–20 mM imidazole can be advantageous).
4. Regeneration solution: 10 mM HEPES, pH 8.3, 150 mM NaCl, 350 mM EDTA, 0.005% Tween-20.
5. Dispensor buffer: 10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% Tween-20.

For the different surfaces an appropriate regeneration solution has to be chosen. An overview of Biacore compatible solutions (e.g., urea, guanidinium hydrochloride, SDS, NaOH) is given under (*1*).

3. Methods

The following methods are described in detail for Biacore systems, however, they are in principle easily transferable to other biosensor devices. A detailed procedure for the most commonly used immobilization strategy, coupling via primary amines, is given below, for other coupling chemistries refer to (*1*).

3.1. Immobilization—Step-By-Step Procedure for Coupling Via Primary Amines

1. Let the sensor chip reach ambient temperature before insertion into the sensor device.
2. Equilibrate the system with running buffer (Run *Prime* procedure on the Biacore instrument).
3. Start a sensorgram and wait until a stable baseline is reached (preferably at a high flow rate between 50 and 100 μ L/min). For CM5 chips, perform an injection of 10–20 mM NaOH for 20–30 s.
4. Switch to the flow cell where the immobilization should take place (if this has not been chosen at the start of the sensorgram). The flow rate should now be set to 5 μ L/min. Again, make sure that the baseline is stable.
5. Perform pre-concentration runs, i.e., perform short injections of ligand at different pH and different concentrations to ensure that electrostatic attraction of protein to the dextran matrix yields a sufficient amount for subsequent immobilization. If this is not the case or if excessive nonspecific binding is observed (i.e., signal does not return to baseline levels after switching to running buffer), try a different immobilization buffer/different pH. It is advisable to change the pH in small increments, i.e., 0.1 pH units.

6. Mix the thawed solutions of NHS and EDC in a 1:1 ratio (50 μ L each).
7. Inject 40 μ L of the mixture (corresponds to 8 min) to activate the CM surface. Select the command *Extraclean* to wash the integrated flow cartridge (IFC) and the needle.
8. Inject the interaction partner until the desired immobilization level is reached. Several injections can be performed.
9. Inject 40 μ L of 1 M ethanolamine hydrochloride, pH 8.5 (corresponds to 8 min) to deactivate excess reactive groups and to remove noncovalently bound material from the surface. Again perform *Extraclean*.
10. Wash the surface(s) with a washing solution, i.e., a regeneration solution that is tolerated by the ligand and monitor if the baseline is stable.
11. The sensor chip can either be used directly or stored at suitable conditions (*see Note 3*). Sometimes it is recommended to run a sensor chip overnight in buffer to assure a stable baseline for the following interaction analysis.

3.2. Kinetic Experiment

1. Insert the sensor chip with immobilized ligand into the Biacore instrument. Make sure that neither side of the chip contains salt deposits or storage solution (you can carefully rinse the chip surfaces with deionized water and soak excess water off the sensor surfaces with a precision wipe placed to one edge on the surface; do not touch the center of the surface containing the immobilized ligand!).
2. Equilibrate the chip in running buffer using the *Prime* procedure.
3. Choose desired flow path.
4. Inject an appropriate dilution of analyte. For kinetic analyses use the *Kinject* command that consumes more analyte, but monitors the dissociation phase without any disturbing peaks caused by needle movements. Note that shifts and bulk effects may occur at the beginning and the end of the injection if the buffer composition of the analyte solution differs from the running buffer. The refractive index of solutions changes dramatically, if even small additional amounts of glycerol, sucrose, detergents or other buffer components are added. This can be overcome by subtracting sensorgrams recorded on reference surfaces. Still, it is highly recommended to match running buffer and analyte buffer as closely as possible, for example, by the use of buffer exchange columns like PD10 or NAP5 (Amersham Biosciences) during analyte preparation.
5. After interaction analysis an appropriate regeneration of the sensor chip surfaces has to be developed to disrupt the analyte-ligand interaction without damaging the biological function of immobilized ligand. For example, a 30 s injection of 10 mM glycine pH 2.2 is suitable for immobilized antibodies. If, in case of antibodies, baseline level is not reached, try longer injections, lower the pH carefully in 0.1 pH steps down to pH 1.9 or alternatively use 0.05% sodium dodecyl sulfate (SDS, note that use of SDS may result in a drifting baseline and that you have to wash the surface for a longer time with running buffer or water).
6. Perform a second injection with the same analyte solution at identical conditions to control for stability of the immobilized ligand during the regeneration procedure. No loss of binding activity should be detected.

7. Once these technical details have been established, a series of experiments with several cycles are started. Biacore systems offer the possibility to write methods for automation; additionally a wizard function is available.

3.3. Data Processing

Raw data need to be processed before the sensorgrams are evaluated. Besides the raw data, on-line referencing in advanced Biacore systems provides data where a control surface is already subtracted. With the Biaevelution software (Biacore AB) rate and equilibrium binding constants can be calculated. The following steps have to be performed before data evaluation (*see Subheading 3.4. and ref. 34*).

1. Zeroing:
 - y-axis: zero the response just prior to the start of the association phase.
 - x-axis: align the starting points of each injection.
2. Reference subtraction:

Correct for refractive index changes and nonspecific binding by subtraction of the reference cell. If the binding curves contain bulk shifts data may be difficult to fit. A software routine is available to detect and subtract bulk refractive index changes, however, you should always verify those data manipulations yourself (*see Subheading 3.2., step 4*).
3. Overlay:

All curves of one data set, i.e., a series of concentrations of one analyte, should be overlayed (after **steps 1** and **2** have been performed). In some instances it might be appropriate to overlay data derived from different ligand surfaces and subtract an additional reference surface (for example, *see Fig. 5*).

3.4. Evaluation of Kinetic Data

Pre-processed data are now ready to be evaluated. Several kinetic modules are available in the Biaevelution software. **References 34** and **35** discuss potential models for data evaluation. Biaevelution supports three ways of data evaluation. A global fit module allows for fitting of an entire set of association and dissociation curves with one set of rate constants which improves the robustness of the fitting procedure. Separate fitting of the association and dissociation phase, respectively, is another option. Furthermore, transient kinetics that are often observed with the binding of small molecules are fitted with equilibrium binding analysis using the equation $Y = B_{\max}X / (K_D + X)$. As an example *see Figs. 6* and *7*.

A 1:1 Langmuir fit model should be applied as a first try (*see Note 4*). However, it is important to consider the biological system first when deciding on the fit model. More complex models of interaction are available. As the complexity of those models increases, the ability to fit the equations to given experimental data will improve automatically! This is simply because there are more degrees of freedom if an increasing number of parameters is applied to

generate a close fit. Therefore, assumptions about the mechanism of interaction should be decided on before applying a more complex model. Complex systems are extremely difficult to interpret, and even sophisticated evaluation software cannot substitute for careful experimental design (see **Fig. 9** and **Subheading 1.3.3.**).

A global fit where one set of rate constants is used for the approximation to the association and dissociation phase should be performed in order to test the reaction model of choice. See **Note 6** for a discussion of pseudo-first order binding kinetics.

The following protocol describes a global fit analysis using BIAevaluation (v3 and higher). For details regarding different models refer to **Note 4**.

1. Open overlayed plot of the processed data from one data set (see **Subheading 3.3.**).
2. Choose *Fit kinetics simultaneous k_a/k_d* (= global fit).
3. Select the injection start and end points as well as the area for the association and dissociation phase. This is simplified with the option *split view* (see **Note 5**).
4. Enter the concentration of analyte for each curve, choose the appropriate model and press *Fit* (for selecting a model see **Note 4**).

3.5. Binding of Warfarin to Serum Proteins: A Practical Approach

In the following the procedures described in **Subheadings 3.1.** to **3.4.** are exemplified in the analysis of the interaction of the low molecular weight compound warfarin, a coumarin derivative, with HSA immobilized to a CM5 chip by amine coupling.

3.5.1. Human Serum Albumin Immobilization

1. Equilibrate a new CM5 sensor chip to room temperature while still enclosed in the nitrogen atmosphere, dock and prime the sensor chip with running buffer (e.g., PBS without DMSO for immobilization).
2. Prepare a solution of 50 $\mu\text{g/mL}$ HSA (essentially fatty acid and globulin free) in 10 mM sodium acetate buffer (pH 5.2).
3. Before activation clean the CM5 sensor surfaces with at least two short pulses (20 s) of 20 mM NaOH and wait until the baseline is stable.
4. Activate the CM5 surface by injecting a freshly prepared 1:1 mixture of NHS (100 mM) and EDC (400 mM) for 8 min at a flow rate of 5 $\mu\text{L/min}$.
5. Inject the HSA solution until a surface density of at least 10.000 RU is reached.
6. Deactivate the HSA surface by injecting 1 M ethanolamine (pH 8.5) for 8 min at a flow rate of 5 $\mu\text{L/min}$.
7. Run the chip overnight in running buffer containing DMSO to achieve a stable baseline.

3.5.2. Dimethyl Sulfoxide Calibration

1. Use freshly prepared, filtered, and degassed running buffer with a well adjusted concentration of organic solvent (DMSO).

2. Prime the IFC and the sensor surfaces at least three times with the appropriate running buffer containing a clearly defined percentage of DMSO.
3. Run a normalize procedure as described for Biacore instruments in order to minimize differences in the refractive indices of the different sensor surfaces followed by another prime procedure.
4. Prepare concentration series of running buffer with different percentages of DMSO, e.g., between 0.5% below and above the DMSO concentration of the running buffer in 0.1% steps.
5. It is very important to avoid evaporation from sample tubes since this will significantly change the buffer composition, in particular the percentage of DMSO.
6. Inject the DMSO concentration series for 1 min each with a flow rate of 30 $\mu\text{L}/\text{min}$ using the *Kinject* command and set report points in the middle of the injection phase.
7. Run each calibration curve in duplicate.
8. Plot the original SPR signal from the reference surface (flowcell 1) in RU vs the reference subtracted SPR signal from the HSA surface (flowcell x-1, see **Fig. 8**).
9. Perform a linear regression for the DMSO calibration curve from which the correction factors for the samples can be calculated.

3.5.3. Interaction Analysis of Low-Molecular-Weight Ligands

1. Dissolve the low-molecular-weight compound completely in 100% DMSO at room temperature.
2. Centrifuge the solution at 16,000g in a tabletop centrifuge for 10 min to remove undissolved constituents.
3. Prepare dilution series of the compound in running buffer and try to match the DMSO concentration exactly to the running buffer. Remember that high refractive index changes induced by varying DMSO concentrations increase the correction factors and thus make the assay less sensitive.
4. Check the solubility of the compound in running buffer at different concentrations. If the compound is not dissolved completely at certain concentrations, centrifuge as described above to remove undissolved constituents but keep in mind that the concentration of compound might be changed significantly by this procedure. Again, it is very important to avoid evaporation.
5. Inject the compound concentration series for 1 min each with a flow rate of 30 $\mu\text{L}/\text{min}$ using the *Kinject* command and set report points in the middle of the injection phase to determine the respective signal.
6. Run at least independent duplicates for each concentration series of compound.
7. Consider to perform a sufficient number of blank injections (running buffer without compound) between the samples in order to prevent sample carry over.
8. Since most small ligand interactions display transient kinetics, a regeneration procedure is usually not necessary to remove bound ligand. However, if the solubility of the compound in running buffer is limited, unspecific binding might occur on the serum protein surfaces. In those cases a regeneration procedure using detergents or mild basic solutions like 10 mM NaOH is usually effective and will not disturb the biological activity of the immobilized protein.

9. Calculate correction factors for each sample by inserting the SPR signal on the reference flowcell (fc1) in the calibration curve as described in the legend to **Fig. 8**. Subtract the respective correction factors from the reference subtracted signal (fc x-1).
10. Plot the corrected SPR signal against the concentration of the compound and perform a nonlinear regression analysis to yield the K_D -value.

4. Notes

1. Sensor surfaces: the following overview is based on the sensor surfaces produced and marketed by Biacore AB, Uppsala, Sweden. Similar surfaces are also employed in other commercially available or custom-built biosensors.

Carboxymethylated (CM-) dextran surfaces: the most widely used sensor surface in BIA. It facilitates coupling of biomolecules via primary amine, sulfhydryl, aldehyde, or carboxyl groups. The dextran matrix provides a hydrophilic environment for the biological interaction to take place. These surfaces are available with dextran polymers of different length and different degrees of carboxymethylation. The shorter the dextrans the lower the overall immobilization capacity of the surface which can be helpful in reducing steric effects, while carboxymethylation affects the charge density of the dextran matrix, which can reduce non-specific binding of positively charged molecules.

Carboxylated surfaces: this surface is devoid of any further modifications besides carboxylation, but supports the same immobilization chemistries as the CM-dextran surfaces. Because of the lack of dextran polymers this surface is more hydrophobic and has a lower immobilization capacity that may be helpful to reduce steric effects when working with high molecular weight ligands.

Streptavidin (SA)-surfaces: Surfaces with pre-immobilized streptavidin molecules allow efficient capturing of biotinylated ligand molecules, ranging from small molecules, DNA, peptides, and proteins to vesicles containing biotinylated lipids. Unlike covalent coupling, the capturing results in an oriented immobilization of the ligand. Electrostatic preconcentration of the ligand on the surface is not necessary.

NTA-surfaces: CM-dextran surfaces derivatized with nitrilotriacetic acid (NTA) for capturing of recombinant proteins with a poly-His tag. The matrix is first loaded with Ni^{2+} , then the poly-His tagged ligand is immobilized to the Ni^{2+} -NTA complex via free coordination sites. After binding analysis, the surface can be regenerated with an injection of EDTA.

Hydrophobic surfaces: Flat hydrophobic surfaces allow lipid vesicles to adsorb directly to the surface, thereby forming a lipid monolayer with the hydrophilic head groups directed towards the soluble phase. Alternatively, dextran surfaces are available modified with lipophilic compounds that permit the immobilization of intact bilayers together with integral membrane proteins.

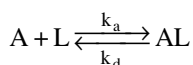
2. Mass transfer limitations: Mass transfer limitation is a phenomenon that occurs when the association and dissociation rate constants are faster than the diffusion rate of the analyte from the laminar flow zone to the relatively undisturbed sur-

face layer. This leads to a depletion of analyte close to the matrix during the association phase, meaning that binding is no longer interaction-controlled but diffusion-limited. The same holds true for dissociation, as the analyte is not transported away fast enough. A related problem is referred to as rebinding since the analyte might rather bind to the ligand than diffuse into the laminar flow zone. This problem may be overcome by injecting soluble ligand during the dissociation phase. Mass transfer limitations slow down both the association and the dissociation rate. It should be noted that these effects are most pronounced with high molecular weight analytes, because of their low diffusion coefficient.

To test for mass transfer effects, perform interaction analyses at different flow rates and with different surface densities. If kinetics look different the interaction may be prone to mass transfer limitations. In addition, a plot of $\ln(dR/dt)$ vs time will display a straight line in mass transfer-limited reactions.

Consequently, mass transfer limitations can be overcome by:

- reduction of the surface density (decreasing available ligand sites on the surface).
 - increasing the flow rate (increasing rate of transfer of the analyte to the surface).
3. Storage conditions: Once a covalent immobilization has been performed, the sensor chip with bound ligand may be taken out of the Biacore instrument and put into a 50 mL screw cap tube filled with approx 35 mL buffer (the sensor surface should be covered). It is not recommended to immerse the chip completely, otherwise the buffer may be contaminated when the chip is taken out and/or the labeling may come off further contaminating the buffer.
 4. 1:1 (Langmuir) binding:



The Langmuir model displays the simplest situation of an interaction between an analyte (A) and an immobilized ligand (L). It is equivalent to the Langmuir isotherm for adsorption to a surface. The Langmuir isotherm was developed by Irving Langmuir in 1916 to describe the dependence of the surface coverage of an adsorbed gas on the pressure of the gas above the surface at a fixed temperature (36,37). The equilibrium that exists between gas adsorbed on a surface and molecules in the gas phase is dynamic, i.e., the equilibrium represents a state in which the rate of adsorption of molecules onto the surface is exactly counterbalanced by the rate of desorption of molecules back into the gas phase. Therefore, it should be possible to derive an isotherm for the adsorption process simply by considering and equating the rates for these two processes. These considerations are also applied to the SPR detection system.

The 1:1 Langmuir module also allows for deviation in the raw data. Sometimes the baseline shows a slight drift that is largely eliminated by the use of a reference cell. However, in analysis with low surface binding capacity (R_{\max} levels

100 RU or less) a model including linear drift may be appropriate (1:1 binding with drifting baseline).

A third 1:1 binding model considering mass transfer limitations is also included in the Biaevaluation software. Thus, kinetic data are produced even though the interaction analysed is mass transfer-limited. Yet, it is recommended to perform the experiment in a way to avoid mass transfer limitations as described in **Note 2** and **ref. 1**.

Alternative models for more complex interaction patterns are available such as bivalent analyte, heterogeneous analyte (competing reactions), heterogeneous ligand (parallel reactions), and two-state reaction (conformational change). Refer to the Biaevaluation (v3.0 or later) manual for details.

It is recommended to use the global fit module for data evaluation. However, for some data sets it is necessary to perform a separate k_a/k_d determination, e.g., if one of the phases is obscured by bulk shifts or if different conditions apply during association and dissociation phase. Biaevaluation also includes a module to fit the association and dissociation phases separately. Additionally, a general fit module including 4-parameter equation, linear fit, solution affinity and steady state affinity is available. More models may be imported into the software.

5. Evaluation with split view: When evaluating a curve set it is important to know which area should be selected for implementing the fit. The Biaevaluation software offers a *split view* function where the plot window is split into two panels with the original curves in the top panel and derivative functions in the bottom panel. Depending on the part of the sensorgram which should be analyzed the user has the option to choose between several mathematical transformations: for the *dissociation phase*: $\ln(dR_0/R_t)$ vs time (termed $\ln(Y_0/Y)$ in Biaevaluation); for the *association phase* $\ln(dR/dt)$ vs time (termed $\ln(\text{abs}(dY/dX))$ in Biaevaluation). This helps to judge whether the model and the parts of the sensorgram selected are appropriate for data evaluation. The functions $\ln(dR/dt)$ and $\ln(dR_0/R_t)$ are linear for 1:1 interactions, constant for mass transfer-limited interactions and curved for more complex systems. It is easier to judge curves in *split view* when the overlay function is turned off. Do not forget to perform the overlay again before proceeding to the next step in the evaluation procedure, i.e., a global fit analysis.
6. The binding of an analyte to a ligand under constant flow is regarded as a pseudo-first order reaction, since the concentration of the analyte is constant in the flow cell. This is not absolutely true, especially with a cuvet system (38,39); the depletion of analyte may have a significant effect on the analyte concentration. The same might also be true for flow systems; as a result of mass transfer limitations the concentration of analyte might be reduced close to the dextran matrix, where interaction with the immobilized ligand takes place (40) (see **Note 2**). This inherent problem may produce the same kind of deviations from pseudo-first order binding processes. Therefore, global fitting may potentially result in conclusions as doubtful as those derived from conventional linear analysis of data (41).

Acknowledgments

The authors would like to thank Claudia Hahnefeld and Oliver Diekmann for valuable input. This work was supported by the Bundesministerium für Bildung und Forschung (BMBF, 031U102F) and the European Union (EU CRAFT QLK2-CT-2002-72419).

References

1. Herberg, F. W. and Zimmermann, B. (1999) Analysis of protein kinase interactions using biomolecular interaction analysis. In *Protein Phosphorylation-A Practical Approach* (Hardie, D. G., ed.), Vol. 2, pp. 335–371. Oxford University Press, Oxford.
2. Stenberg, E., Persson, B., Roos, H. and Urbaniczky, C. (1991) Quantitative determination of surface concentration of protein with surface plasmon resonance using radiolabeled proteins. *J. Colloid Interface Sci.* **143**, 513–526.
3. Piehler, J., Brecht, A., Geckeler, K. E., and Gauglitz, G. (1996) Surface modification for direct immunoprobes. *Biosens. Bioelectron.* **11**, 579–590.
4. Piehler, J., Brecht, A., and Gauglitz, G. (1996) Affinity detection of low molecular weight analytes. *Analytical Chemistry* **68**, 139–143.
5. Piehler, J., Brecht, A., Gauglitz, G., Zerlin, M., Maul, C., Thiericke, R., and Grabley, S. (1997) Label-Free Monitoring of DNA-Ligand Interactions. *Analytical Biochemistry* **249**, 94–102.
6. Birkert, O. and Gauglitz, G. (2002) Development of an assay for label-free high-throughput screening of thrombin inhibitors by use of reflectometric interference spectroscopy. *Anal. Bioanal. Chem.* **372**, 141–147.
7. Birkert, O., Tunnemann, R., Jung, G., and Gauglitz, G. (2002) Label-free parallel screening of combinatorial triazine libraries using reflectometric interference spectroscopy. *Anal. Chem.* **74**, 834–840.
8. Kröger, K., Bauer, J., Fleckenstein, B., Rademann, J., Jung, G., and Gauglitz, G. (2002) Epitope-mapping of transglutaminase with parallel label-free optical detection. *Biosensors and Bioelectronics* **17**, 937 – 944.
9. Hanel, C. and Gauglitz, G. (2002) Comparison of reflectometric interference spectroscopy with other instruments for label-free optical detection. *Anal. Bioanal. Chem.* **372**, 91–100.
10. Wink, T., de Beer, J., Hennink, W. E., Bult, A., and van Bennekom, W. P. (1999) Interaction between plasmid DNA and cationic polymers studied by surface plasmon resonance spectrometry. *Analytical Chemistry* **71**, 801–805.
11. Melendez, J., Carr, R., Bartholomew, D. U., Kukanskis, K., Elkind, J., Woodbury, R., Furlong, C., and Yee, S. (1996) A commercial solution for surface plasmon sensing. *Sensors and Actuators B: Chemical* **35**, 212–216.
12. Cush, R., Cronin, J., Steward, W., Maule, C., Molloy, J., and Goddard, N. (1993) The resonant mirror: a novel optical biosensor for direct sensing of biomolecular interactions, Part I: Principle of operation and associated instrumentation. *Biosens. Bioelectron.* **8**, 347–354.

13. Cunningham, B., Li, P., Lin, B., and Pepper, J. (2002) Colorimetric resonant reflection as a direct biochemical assay technique. *Sensors and Actuators B: Chemical* **81**, 316–328.
14. Baird, C. L. and Myszka, D. G. (2001) Current and emerging commercial optical biosensors. *J. Mol. Recognit.* **14**, 261–268.
15. Rich, R. L. and Myszka, D. G. (2003) A survey of the year 2002 commercial optical biosensor literature. *J. Mol. Recognit.* **16**, 351–382.
16. Zimmermann, B., Hahnefeld, C., and Herberg, F. W. (2002) Applications of biomolecular interaction analysis in drug development. *TARGETS* **1**, 66–73.
17. Engvall, E. (1980) Enzyme immunoassay ELISA and EMIT. *Methods Enzymol.* **70**, 419–439.
18. Karlsson, R. (1994) Real-time competitive kinetic analysis of interactions between low-molecular-weight ligands in solution and surface-immobilized receptors. *Anal. Biochem.* **221**, 142–151.
19. Dillon, P. P., Daly, S. J., Manning, B. M., and O’Kennedy, R. (2003) Immunoassay for the determination of morphine-3-glucuronide using a surface plasmon resonance-based biosensor. *Biosensors and Bioelectronics* **18**, 217 – 227.
20. Tudos, A. J., Lucas-van den Bos, E. R., and Stigter, E. C. (2003) Rapid surface plasmon resonance-based inhibition assay of deoxynivalenol. *J. Agric. Food Chem.* **51**, 5843–5848.
21. Deinum, J., Gustavsson, L., Gyzander, E., Kullman-Magnusson, M., Edström, Å., and Karlsson, R. (2002) A thermodynamic characterization of the binding of thrombin inhibitors to human thrombin, combining biosensor technology, stopped-flow spectrophotometry, and microcalorimetry. *Analytical Biochemistry* **300**, 152–162.
22. Day, Y. S., Baird, C. L., Rich, R. L., and Myszka, D. G. (2002) Direct comparison of binding equilibrium, thermodynamic, and rate constants determined by surface- and solution-based biophysical methods. *Protein Sci.* **11**, 1017–1025.
23. Roos, H., Karlsson, R., Nilshans, H., and Persson, A. (1998) Thermodynamic analysis of protein interactions with biosensor technology. *J. Mol. Recognit.* **11**, 204–210.
24. Markgren, P. O., Schaal, W., Hamalainen, M., Karlen, A., Hallberg, A., Samuelsson, B., and Danielson, U. H. (2002) Relationships between structure and interaction kinetics for HIV-1 protease inhibitors. *J. Med. Chem.* **45**, 5430–5439.
25. Karlsson, R., Kullman-Magnusson, M., Hamalainen, M. D., Remaeus, A., Andersson, K., Borg, P., Gyzander, E., and Deinum, J. (2000) Biosensor analysis of drug-target interactions: direct and competitive binding assays for investigation of interactions between thrombin and thrombin inhibitors. *Anal. Biochem.* **278**, 1–13.
26. Gestwicki, J. E., Hsieh, H. V., and Pitner, J. B. (2001) Using receptor conformational change to detect low molecular weight analytes by surface plasmon resonance. *Anal. Chem.* **73**, 5732–5737.
27. Carrasco, C., Facompre, M., Chisholm, J. D., Van Vranken, D. L., Wilson, W. D., and Bailly, C. (2002) DNA sequence recognition by the indolocarbazole antitu-

- mor antibiotic AT2433-B1 and its diastereoisomer. *Nucl. Acids. Res.* **30**, 1774–1781.
28. Hendrix, M., Priestley, S. E., Joyve, G. F., and Wong, C.-H. (1997) Direct observation of aminoglycoside-RNA interactions by surface plasmon resonance. *J. Am. Chem. Soc.* **119**, 3641–3648.
29. Chapman, R. L., Stanley, T. B., Hazen, R., and Garvey, E. P. (2002) Small molecule modulators of HIV Rev/Rev response element interaction identified by random screening. *Antiviral Res.* **54**, 149–162.
30. Li, K., Davis, T. M., Bailly, C., Kumar, A., Boykin, D. W., and Wilson, W. D. (2001) A heterocyclic inhibitor of the REV-RRE complex binds to RRE as a dimer. *Biochemistry* **40**, 1150–1158.
31. Cheng, X., Phelps, C., and Taylor, S. S. (2001) Differential binding of cAMP-dependent protein kinase regulatory subunit isoforms Ialpha and Ibeta to the catalytic subunit. *J. Biol. Chem.* **276**, 4102–4108.
32. Rich, R. L., Day, Y. S., Morton, T. A., and Myszk, D. G. (2001) High-resolution and high-throughput protocols for measuring drug/human serum albumin interactions using BIACORE. *Anal. Biochem.* **296**, 197–207.
33. Yaqub, S., Abrahamsen, H., Zimmerman, B., Kholod, N., Torgersen, K. M., Mustelin, T., Herberg, F. W., Tasken, K., and Vang, T. (2003) Activation of C-terminal Src kinase (Csk) by phosphorylation at serine-364 depends on the Csk-Src homology 3 domain. *Biochem. J.* **372**, 271–278.
34. Myszk, D. G. (2000) Kinetic, equilibrium, and thermodynamic analysis of macromolecular interactions with BIACORE. *Methods Enzymol.* **323**, 325–340.
35. Karlsson, R. and Falt, A. (1997). Experimental design for kinetic analysis of protein-protein interactions with surface plasmon resonance biosensors. *J. Immunol. Methods* **200**, 121–133.
36. Langmuir, I. (1916) The constitution and fundamental properties of solids and liquids. Part I. Solids. *J. Am. Chem. Soc.* **38**, 2221–2295.
37. Langmuir, I. (1918) The adsorption of gases on plane surfaces of glass, mica and platinum. *J. Am. Chem. Soc.* **40**, 1361–1403.
38. Hall, D. R., Gorgani, N. N., Altin, J. G., and Winzor, D. J. (1997) Theoretical and experimental considerations of the pseudo-first-order approximation in conventional kinetic analysis of IAsys biosensor data. *Anal. Biochem.* **253**, 145–155.
39. O’Shannessy, D. J. and Winzor, D. J. (1996) Interpretation of deviations from pseudo-first-order kinetic behavior in the characterization of ligand binding by biosensor technology. *Anal. Biochem.* **236**, 275–283.
40. Hall, D. R., Cann, J. R., and Winzor, D. J. (1996) Demonstration of an upper limit to the range of association rate constants amenable to study by biosensor technology based on surface plasmon resonance. *Anal. Biochem.* **235**, 175–184.
41. Schuck, P. and Minton, A. P. (1996) Analysis of mass transport-limited binding kinetics in evanescent wave biosensors. *Anal. Biochem.* **240**, 262–272.



<http://www.springer.com/978-1-58829-372-5>

Protein-Ligand Interactions

Methods and Applications

Nienhaus, G.U. (Ed.)

2005, XI, 568 p., Hardcover

ISBN: 978-1-58829-372-5

A product of Humana Press