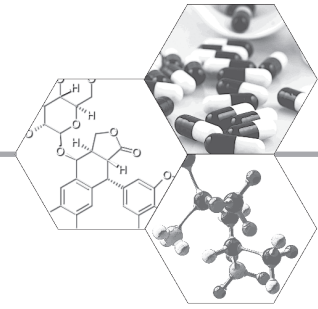

Integrating surface plasmon resonance biosensor-based interaction kinetic analyses into the lead discovery and optimization process

Surface plasmon resonance biosensor technology has come of age and become an important tool for drug discovery. It is a label-free biophysical technique for the kinetic analysis of molecular interactions that provides exceptionally information-rich data. Recent improvements in sensitivity, experimental design, data analysis and sample throughput makes it suitable for use throughout the drug-discovery process. This article outlines the use of SPR biosensor technology for small-molecule drug discovery and exemplifies how it complements other techniques. The technology is especially valuable for fragment-based lead discovery since it has the required sensitivity and throughput for screening of fragment libraries. Hits can be identified with respect to multiple criteria, defined by the experimental design used for screening. Expansion of hits and subsequent characterization and optimization of leads can be performed with a variety of experiments exploiting the kinetic resolution of the technology. Leads identified by this strategy can therefore be extensively characterized with respect to their interactions, with their target as well as with nontarget proteins. Although it may take some time for the methods to become well established, and for the research community to reach proficiency and fully embrace the information-rich data that can be obtained, it can be predicted that this technology will be widely used for drug discovery within the near future. It is expected that the technology will be particularly important for fragment-based strategies and integrated with other experimental technologies as well as with computational methods.

The application of surface plasmon resonance (SPR) biosensor technology for various areas of basic research and drug discovery is a subject that is gaining increasing interest. The first instrument was launched by Biacore less than 20 years ago [1] and a range of commercial biosensors, based on SPR and other detection methods, is available today. Although the instruments and technologies may appear similar, their performance and suitability for different types of studies varies, as has been illustrated in a global benchmark study that has compared the performance of affinity-based sensors [2].

In contrast to most methods for interaction analysis, SPR biosensor experiments can provide the kinetic details of interactions. This is a consequence of the time resolution of the analysis, which allows individual kinetic rate constants, as well as affinities, to be determined. The technology can even reveal the characteristics of more complex mechanisms (e.g., involving multiple steps or conformations of the target or ligand) as well as the energetics and forces involved. Many excellent and extensive reviews on this topic have already been published [3,4] and publications in the area are outlined in yearly surveys; see, for example, the survey for 2007 [5]. The present review focuses on recent developments in the field, in particular those of relevance for small-molecule and fragment-based lead discovery [6].

SPR biosensor basics

The basic principles of SPR biosensor technology and the information that can be obtained are sometimes difficult to grasp since there are several key features that each need to be understood. This technical background is focused on the methodology implemented in the range of Biacore instruments designed for the analysis of low-molecular-weight analytes. These are today, to the best of my knowledge, the only instruments with adequate sensitivity, time resolution and flexibility in experimental design for small-molecule drug discovery.

Due to the rapid development of SPR biosensor technology, the extent of what can actually be achieved today is not widely understood. In

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addition, there are some remaining misconceptions from the early days of SPR biosensors which make some researchers hesitant to utilize the technology for their areas of interest. This misconception primarily concerns the sensitivity of measurements and the quality of data. In order to put the application of SPR biosensor analysis into perspective and to encourage more researchers to use the technology, especially for low-molecular-weight drug discovery, a brief description of the technology is warranted.

■ Detection of interactions using SPR biosensors

Surface plasmon resonance biosensors can be seen as flat affinity chromatography surfaces, which, in real time, sense the interaction between immobilized molecules and analytes injected into the mobile phase. SPR is an optical phenomenon that is associated with the total internal reflection of light at the boundary between two media of different optical properties, described by their different dielectric functions [7]. In the SPR biosensor, plane-polarized light is reflected by a thin gold film on a sensor chip that is positioned in a microfluidic system [8,9]. An advantage of this is that it does not require labeling of any of the interactants or reporter molecules. The light never passes through the sample, and there is therefore no absorption or dispersion of light. The signal is influenced by the small changes in refractive index that can be induced by molecules close to the surface and is recorded in refractive (or resonance) units (RUs). Interactions between low-molecular-weight analytes and immobilized proteins are often thought to be undetectable as there is a correlation between the magnitude of the signal and the change in mass upon the binding of an analyte to the surface. However, although the change in refractive index at the surface is associated with the mass of the analyte, current instruments have the sensitivity required for analysis even of small molecules. Nonetheless, there are other effects that influence the refractive index and may be significant in experiments involving low-molecular-weight analytes [10]. The advantage of the general detection principle may thus become a challenge when the signal is to be interpreted mechanistically. SPR biosensor-based experiments therefore require appropriate and extensive references and controls, as well as cautious **mechanistic interpretation** of results, especially for more complex interactions.

Surface plasmon resonance biosensor

Type of biosensor where the interaction between two molecules is detected by the optical phenomenon of surface plasmon resonance. It is a label-free technique that is implemented in various types of biosensors with different experimental set-ups and data output

Mechanistic characterization

The determination of the species and steps involved in an interaction. The analysis is qualitative and is the basis for defining the reaction scheme and equations describing the complete interaction mechanism

■ Sensor surface preparation

The quality of experiments is dependent on the ability to make highly functional and stable sensor surfaces. The metal film that is a necessary feature of the SPR detection system is therefore modified, so that the preparation of sensor surfaces is facilitated and the protein is immobilized in a suitable environment. The most popular sensor chips are covered by a thin layer of dextran that has been functionalized with carboxyl groups, allowing covalent anchoring of the protein (or other molecule) of interest [11]. Other specialized surfaces for immobilization of biomolecules can be prepared, such as self-assembled monolayers with small-molecule ligands covalently linked to thiols [12]. There are many chemical strategies for immobilizing the proteins directly to the dextran surfaces [13–15] and a variety of tags and fusion proteins are becoming popular [16–18]. This flexibility in sensor surface preparation allows the selection of an immobilization strategy with respect to the characteristics of the protein and the types of experiments to be performed. The repertoire of available immobilization techniques should be exploited and evaluated when an assay for a new target is defined and optimized, since it is essential that the immobilized protein is fully functional and that the binding site of interest is exposed to the buffer flowing over the surface. Moreover, when low-molecular-weight analytes are studied, there are often extensive nonspecific interactions between the compounds and the sensor matrix or the fusion protein used for immobilization (e.g., glutathione *S*-transferase). It may, therefore, be necessary to test alternative immobilization procedures, even if the functionality of the protein itself is not an issue.

The quality of the protein sample used to prepare the sensor surface is also crucial for good results. It should not only be pure, but also available at high concentrations in a buffer amenable to the immobilization procedure being used. For example, in order for the protein to be efficiently immobilized via amine coupling, the most common immobilization chemistry, it should be provided in a buffer with a pH at least one unit below its pI. The net positive charge of the protein under such conditions attracts it to the negatively charged sensor matrix, facilitating the immobilization of large amounts of protein. This is not possible for acidic proteins, which are therefore better immobilized by other techniques. The need for a high local

concentration of protein in the matrix is also the underlying reason why proteins should be provided at high concentrations and why impurities may reduce the quality of sensor surfaces even if they are inert in the measurements.

The quality of a sensor surface is not only dependent on the amount of immobilized protein, but also its degree of functionality, which is often surprisingly low even in the sample used for immobilization [19]. It can be determined after immobilization by comparing the maximum 'apparent binding capacity' of a surface, estimated from the signal at saturation for a reference compound with adequate affinity for the immobilized protein, with the 'theoretical binding capacity', calculated from the molecular weights of the protein and the reference compound and the amount of immobilized protein. Due to the high sensitivity required and the relatively large amounts of protein that must be immobilized for the analysis of low-molecular-weight analytes (especially fragments), these issues can be critical.

A related aspect that influences the possibility of performing high-quality experiments is the stability of the protein on the chip. Many proteins lose functionality over time, due to aggregation, partial unfolding or even autohydrolysis, as in the case of proteases [20,21]. By using conditions optimized for protein stability [21], stabilized mutants or chemically crosslinking the protein [20], this problem can be reduced. However, when analyzing high-affinity analytes, it is sometimes necessary to use relatively harsh surface-regeneration conditions in order to perform new experiments on the surface. On occasions, these conditions damage the protein, accelerating the aging of a sensor surface. At times, it is not possible to find regeneration conditions that do not destroy the surface and repeated experiments can therefore not be performed on the same chip [ELINDER M *ET AL.* INHIBITION OF RESISTANT HIV-1 BY MIV-170, A SLOWLY DISSOCIATING NON-NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITOR, MANUSCRIPT SUBMITTED]. It is expected that these problems concerning preparation of sensitive and stable sensor surfaces will be reduced in the future with the introduction of a greater repertoire of protein-production and immobilization techniques and the availability of a wider range of protein tags. These should be developed with respect to their ability to immobilize large amounts of functional protein on the surface, ideally via a method that allows the protein to be removed and new protein to be captured on the same chip.

■ Experimental design & data analysis

The experimental design needs to be suitable for the purpose, and methods of determining the most efficient experimental design for SPR biosensor experiments have been presented [22,23]. The standard experimental mode for analysis of ligand–target interactions is the immobilization of the protein and injection of the low-molecular-weight compound as analyte, as assumed in the text above and illustrated in **FIGURE 1**. The reason for this is that a large number of analytes can be analyzed with the same sensor surface, which is typically what is needed for hit and lead identification, and for optimization. Although the inverse experiment can be performed (i.e., where the ability of the protein to interact with an immobilized ligand in the presence or absence of a test compound is measured), this indirect method is generally less informative, but can be useful for some applications. Competition

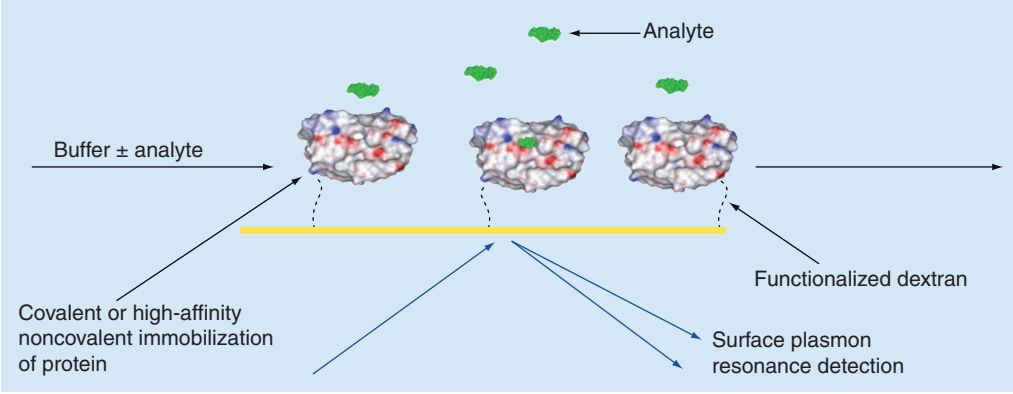

Figure 1. Principles of surface plasmon resonance biosensor analysis of molecular interactions.

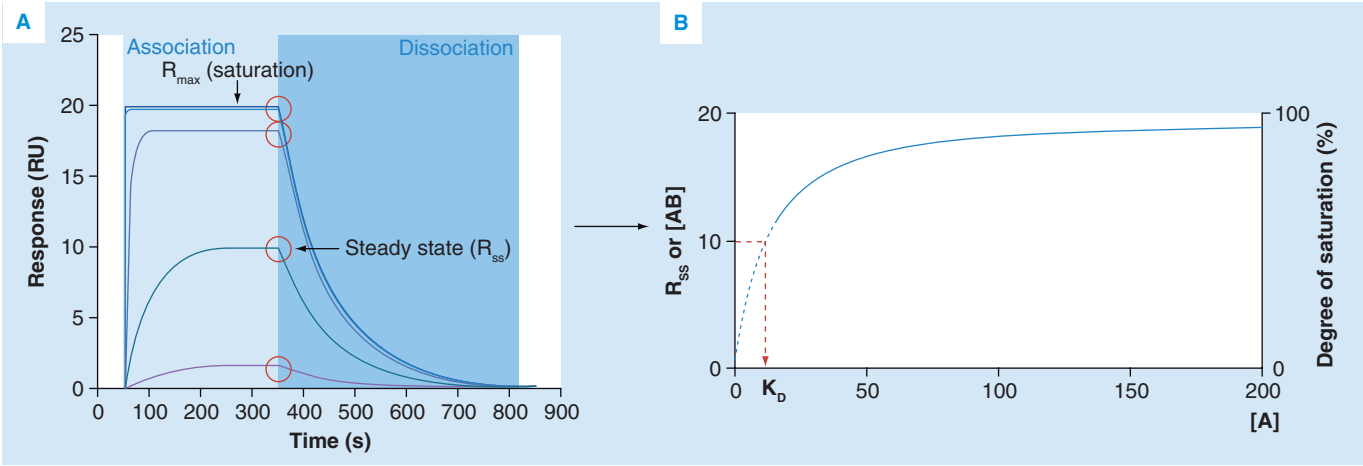

Figure 2. Relationship between progress curves for association and dissociation of analytes interacting with (A) a sensor surface (sensorgrams) and (B) steady-state signals (R_{ss}) as a function of analyte concentration. Simulated data for a simple 1:1 interaction.

Interaction mechanism

Mechanistic model for an interaction visualized by a reaction scheme that specifies all definable species and steps involved in the interaction. The simplest interaction model is a 1:1 Langmuir model, described by:

$$A + B \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} AB$$

where the association and dissociation rate constants (k_{on} and k_{off}, respectively), the two interacting molecules (A and B) and the resulting complex (AB) are defined. It can be expressed in the form of a mathematical equation:

$$[AB] = \frac{[A] \times [B]_{tot}}{K_D + [A]}$$

where the equilibrium dissociation constant is defined as:

$$K_D = \frac{[A] \times [B]}{[AB]} = \frac{k_{off}}{k_{on}}$$

Screening

The procedure for searching through large collections of samples for compounds that fulfill defined characteristics. The procedure is primarily optimized for high-throughput analysis

experiments are often useful, even when the protein is immobilized, as a complement to the direct-binding analysis. In addition to the basic features of experimental design, there are a number of parameters that can be varied. This is the basis for the highly information-rich data that can be obtained by this technique.

The type of data that is obtained and the method used for analysis depends on the type of experiment and the required information. There are three principal levels of data analysis:

- High-resolution analysis: direct analysis of progress curve data representing the complete time course for association and dissociation, ideally for several concentrations of analyte **(Figure 2A)**. This type of data can be used to define the mechanism of the interaction and determine the corresponding kinetic rate constants.
- Medium-resolution analysis: analysis of equilibrium data extracted from the steady-state signals of progress curves obtained with a series of different analyte concentrations **(Figure 2A & B)**. The data can be used to estimate the basic interaction mechanism and equilibrium parameters (i.e., stoichiometry and affinity [K_D]). It is often used when kinetic rate constants are too fast or slow to be quantified.
- Low-resolution analysis: report point-based data extracted at one or several defined times during or after injection, not necessarily at steady state. By comparing the signal for test compounds to those for positive and negative references, it is possible to conclude whether an analyte interacts with the protein or not. It is an efficient method that is often sufficient for screening.

Figure 2 illustrates the difference between complete progress curves for interactions **(Figure 2A)** and the commonly used graph for equilibrium-based data of complex formation as a function of ligand concentration **(Figure 2B)**. Report points extracted during the steady state phase in the progress curves are used to generate the saturation curve. A good understanding of the mathematical principles of data analysis and the equations to be used is required for extraction of meaningful information from biosensor experiments. Much of the analysis is taken care of by the software accompanying the instrument. In order to fully exploit the information potential of SPR biosensor experiments, more extensive experimental designs for high-resolution analysis are required.

State-of-the-art

■ Sensitivity

In the early days of SPR biosensors, the sensitivity of measurements only allowed the analysis of interactions with macromolecular analytes. Consequently, when interactions with low-molecular-weight compounds (i.e., drug-like compounds) were studied, they were immobilized and the macromolecule was used as an analyte. Although such assays still have their uses, they are no longer necessary to obtain sufficient signals.

Today, the sensitivity allows detection not only of low-molecular-weight analytes but also of ions, provided that their binding is associated with a major conformational change [24]. Ligands

often give rise to conformational changes in the protein upon binding, but even changes in the environment (e.g., pH) can induce conformational changes [24–28]. In most techniques, such structural changes go unnoticed, however with SPR biosensors they can sometimes be detected. Mechanistic interpretations are possible, providing that extensive controls have been carried out and that alternative evidence is available. **Figure 3** illustrates the conformational change of human phenylalanine hydroxylase upon binding of L-phenylalanine. The same results were obtained using intrinsic tryptophan fluorescence analysis **(Figure 3A)** and SPR biosensor analysis **(Figure 3B)**.

An example of the ability to detect ion interactions is the binding of Ca^{2+} to C-reactive protein (CRP). It is associated with large rearrangements in a loop. In this case, it was possible to determine the affinities of the two calcium ions [29]. The sensorgrams for this interaction are shown in **Figure 4**.

The sensitivity limitations of the technology today are therefore not typically restricted by the mass of the analyte but primarily related to the possibility of immobilizing the protein in a functional and stable form at sufficient concentrations for sensitive measurements.

■ Information content

Another aspect of sensitivity concerns the type of information that can actually be extracted when the interaction yields detectable signals. It is dependent on the experimental design and the type of data analysis used. The range of rate constants and affinities that can be determined today has been well described and the methodological aspects considered [22]. In principle, the rate of diffusion and the ability to distinguish an irreversible interaction from one with a very slow dissociation sets the limits for the rate constants that can be determined. Affinities can typically be determined if it is possible to study the interactions with analyte concentrations that are significantly higher than the K_D value. This, therefore, depends on the solubility of the analyte. For interactions that are more complex than simple 1:1 interactions (e.g., those involving multiple binding sites or conformational changes), the requirement for high-quality data over a wide range of concentrations (including concentrations > K_D^{app}, i.e., the apparent K_D) alters the limits of values and the parameters that can be reliably quantified. Moreover, the availability of references (i.e., reference compounds and proteins) is essential for the validation of results and determines the reliability of the interpretations. More complex data require a realistic mechanistic model that can be confirmed by other types of experiments or computational modeling. In the case of the interaction between L-phenylalanine and human phenylalanine hydroxylase **(Figure 3)**, the rate constants were not reported [28], presumably due to the difficulties in determining parameters for complex models. Similarly, the interaction between Ca^{2+} and CRP **(Figure 4)** was only quantifiable

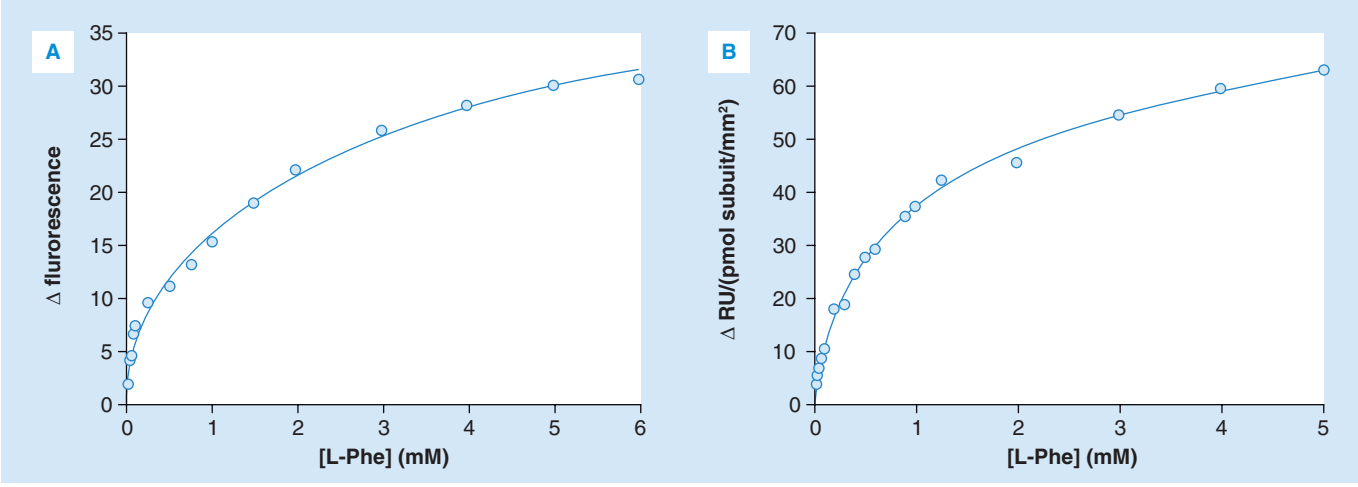

Figure 3. Experimental evidence for the induction of a conformational change in human phenylalanine hydroxylase upon the binding of L-phenyl alanine. Steady-state equilibrium response determined by **(A)** intrinsic tryptophan fluorescence and **(B)** by surface plasmon resonance. Both experiments are well described by a model accounting for a conformational change (solid lines).
RU: Refractive units.
Reproduced with permission from [28] © the Biochemical Society.

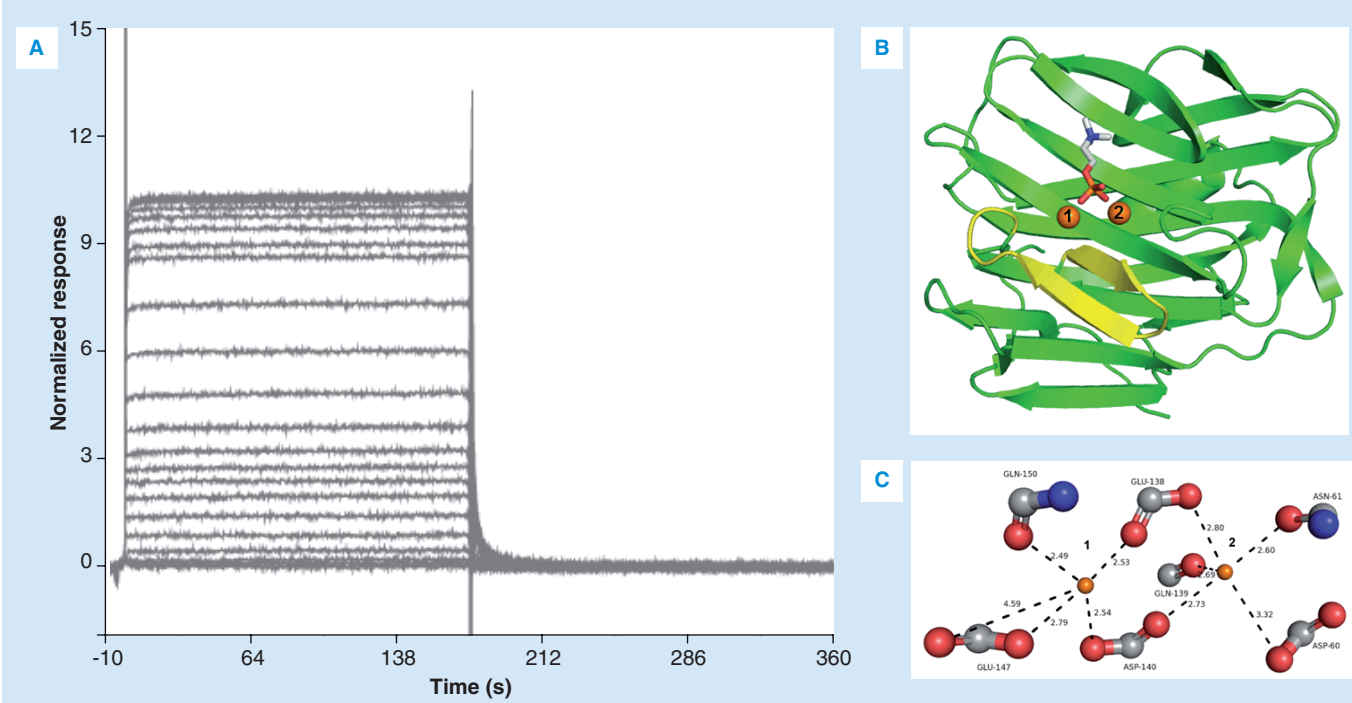

Figure 4. (A) Sensorgrams for the interaction between Ca^{2+} and C-reactive protein with a dilution series of Ca^{2+} starting at 50 mM. **(B)** Structure of C-reactive protein with bound phosphocholine and Ca^{2+} ions and **(C)** close-up view of the coordination of the two Ca^{2+} ions.
Reproduced with permission from [29].

in terms of affinities due to the rapid association and dissociation rates. This illustrates the difference between the sensitivity required to detect an interaction or its effects on the target and the ability to extract all the information regarding the phenomenon, especially in quantitative terms.

An additional form of information that can be obtained is related to the strange sensorgrams that arise as a consequence of aggregation, micelle formation or precipitation. In contrast to many other methods, these are often seen directly in the raw data, especially at high concentrations of analyte [30]. This information can be very important, particularly in identifying false positives and understanding the reasons behind false negatives. However, in order to reduce the risk of misinterpretations, compounds are typically analyzed in 5% DMSO at concentrations below those where any problems with solubility are expected. Concentrations can be up to 500 µM or even 1 mM; however, many compounds can only be studied at much lower concentrations.

■ Material consumption

In comparison to many other techniques for ligand–target interaction analysis, SPR biosensor experiments consume relatively low amounts of protein. The validation of a fragment library of 930 compounds, using a typical experimental design for screening with a single concentration of compounds, required 0.025–0.8 mg of protein, depending on the protein [31]. The screening of a focused library for inhibitors against HIV reverse transcriptase (RT) consumed less than 0.5 mg of each of the four enzyme variants used for screening [32]. The factors that influence protein consumptions are, primarily, protein stability and the relative amount of functional protein in the preparation used for immobilization. Less material will be required for characterization studies where fewer experiments (i.e., fewer immobilizations and compound injections) are required [22].

■ Throughput

The throughput varies according to the flow cell design of the instrument used, which is one of the features that differs between different instrument models. The flow cell design influences the experimental strategy and how efficiently the sensor surfaces can be used. Today, the highest throughput can be obtained with the Biacore A100 instrument, which has an array format that allows four to 16 different proteins to be screened in parallel **(FIGURE 5)** [33].

The throughput is also dependent on the stability of the protein and other practical factors concerning the experimental setup. For example, the screening of the same panel of proteins in all four flow cells typically allows 960 compounds (including references) to be screened at single concentrations per 16 h [Lindgren M, Beactica AB, Uppsala, Sweden, Pers. Comm], while 1400 samples (including references) could be screened at six concentrations in 22 h [Winquist J, Uppsala University, Uppsala, Sweden, Pers. Comm.]. An often overlooked aspect of throughput is the extensive time required for handling of the acquired data, a task that can be more time consuming than the experiments themselves. Recent improvements in the software for analysis of data from SPR biosensors has automated many of the steps; however, a reliable analysis requires an experienced user with a good understanding of the type of analysis that is appropriate for each experiment. Many users complement the instrument-dedicated software with external programs for analysis and/or data visualization, such as Spotfire [32], MatLab [32] or Scrubber [30].

■ Alternative techniques

The range of commercial biosensors available today differ in their sensor surface design, detection principles and sensitivity. This influences both their cost and capacity to be used for different types of studies. For macromolecular interactions (e.g., antibody–antigen interactions), the range of suitable instruments is larger than for analysis of interactions with low-molecular-weight analytes, which is considerably more challenging [2]. The alternative techniques for interaction studies involving low-molecular-weight ligands are therefore based on other principles and give important complementary information. For example, NMR, x-ray crystallography and computational modeling techniques are all useful for obtaining structural details of interactions. Microcalorimetry is commonly used for thermodynamic studies [34], while fluorescence-based thermal shift assays [35] are gaining in popularity for use in screening. However, these techniques also have their limitations. For example, NMR and x-ray crystallography suffer from a considerably larger protein consumption than SPR biosensor analysis and the time required to perform microcalorimetry experiments is sometimes too long with respect to the stability of the protein. Moreover, x-ray crystallography is obviously limited to studying proteins that are readily crystallized. There are currently no ideal techniques for the study of small-molecule interactions with membrane-bound proteins, although the research in this area is intense. Since there are no *a priori* limitations to SPR biosensor analysis of such proteins and progress in the area is being made [36–38], it is expected that this technology will be important for studies of membrane-bound proteins. The increasing popularity of biophysical assays for studies of protein–ligand interactions, partly spurred by fragment-based drug discovery, is forcing continued development of these types of techniques and applications. It is therefore expected that researchers will have a greater range of instruments to chose from in the near future and that these intruments will be better suited to the requirements for small-molecule drug discovery.

Thermodynamic characterization

Quantitative determination of the energetics of an interaction mechanism (i.e., change in free energy [ΔG], enthalpy [ΔH] and entropy [ΔS]). Using surface plasmon resonance biosensor analysis, these can be determined for the equilibrium as well as for any individual steps that can be resolved (i.e., association and dissociation)

SPR biosensors in lead discovery

Until recently, SPR biosensors have primarily been suited for secondary screening and lead optimization [39]. However, due to increased throughput and higher sensitivity, this is no longer the case.

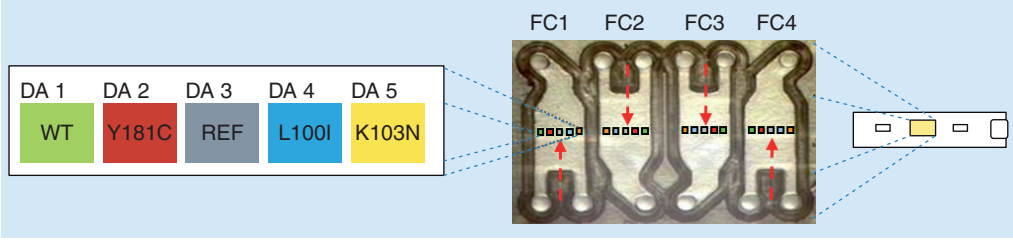

Figure 5. The use of four DAs in each of the four FCs of a sensor chip in a Biacore A100 sensorgrams for screening against four variants of HIV reverse transcriptase. With this setup, four compounds can be screened in parallel against four different proteins.
DA: Detection area; FC: Flow cells.
Reproduced with permission from [32].

Screening & hit identification

As a result of the information-rich screening data that is obtained, the compounds that give a positive signal can be ranked and prioritized on the basis of the desired type of compound for a particular drug-discovery endeavor. Methods for screening of small, focused compound libraries have therefore developed from simply identifying compounds that interact with the target [40], to protocols that identify hits with a certain selectivity [41] or a specific kinetic profile [32]. The latter study describes how identification of compounds with slow dissociation from the wild-type and three resistant target variants of HIV-1 RT were useful as selection criteria when screening a compound library resulting from the optimization of non-nucleoside reverse transcriptase inhibitor (NNRTI) leads against the wild-type enzyme. This screen identified a compound previously not regarded as of interest, since its characteristics were similar to other compounds when a simpler hit identification criterion had been used **(FIGURE 6)**.

Experimental designs that identify compounds binding to a particular site [42] or exclude compounds interacting unselectively have also been published [21]. Several hit criteria were used in the second study in order to identify compounds binding to the active site of MMP-12. By using carbonic anhydrase as a reference, compounds that bound indiscriminately to an active site containing a metal ion could be excluded. This illustrates that the high sensitivity of an SPR biosensor assay can be used to its full potential by including multiple selection filters already at the stage of screening. Another advantage of the high sensitivity of measurements using SPR biosensors is that the technology also allows the screening of fragment libraries [21,42].

Fragment-based lead discovery

Fragment-based lead discovery is one of the more interesting developments in the area of drug discovery lately (for a review, see [6]) and is an area in which SPR biosensor analysis is expected to be of significant importance. It was initially a strategy based on NMR [43] but was later followed by x-ray crystallography [44]. It can now also be achieved by SPR [21,42] and other optical techniques [4]. The principle for this strategy is that libraries of low molecular weight compounds (<300 Da) are screened with a method that can detect low-affinity interactions. Since fragments are unlikely to exhibit high-affinity interactions with the target, the concept of ligand efficiency, expressed as:

$$\Delta g = \frac{\Delta G}{N_{nonhydrogen\ atoms}}$$

where

$$\Delta G = -RT \times InK_D$$

is used to identify compounds with the potential to be developed into high-affinity leads with characteristics suitable for drug candidates [45]. Ligand efficiency can be interpreted as the affinity normalized with respect to the molecular weight of the compound. It compensates for the low affinity of fragments for the few available atoms and groups that can contribute to the interaction. By prioritizing hits with respect to ligand efficiency rather than affinity, it is expected that the optimized leads will be easier to evolve into drugs.

Fragment libraries have several advantages over conventional compound libraries. One is a consequence of their small size, which makes them better suited to exploring binding sites (cavities) on protein surfaces [46]. Since the size of chemical space correlates with complexity of compounds, a well-designed fragment library, consisting of only a few thousand compounds, can cover a sufficient area of chemical space to identify scaffolds for lead discovery. Such libraries are rapid to screen and very well suited to the flexible experimental design that can be adopted with SPR biosensors, for example involving parallel screening of multiple targets or competition screening [21].

A crucial step in fragment-based drug discovery is the expansion of low-affinity fragments into high-affinity leads. Fragment hits are relatively easily converted into leads with higher affinity by adding groups or by linking with other fragments binding to an adjacent region in the binding site. Once high-affinity leads have been obtained, they are optimized by the same procedures as leads identified by other strategies.

The challenge for fragment screening is related to being able to perform analysis with high sensitivity. Moreover, information beyond 'percentage binding relative to a reference compound' is not always possible to obtain, since the concentrations that can be used are often not above the affinities of the interactions [22]. Even for expanded fragments with higher affinity, the information is often limited to affinity as they typically have rapid interaction kinetics, outside the quantifiable range [47]. Despite the challenges, SPR biosensor

analysis has successfully been implemented for various tasks in fragment-based discovery. It has, for example, been used for identifying or characterizing inhibitors of hepatitis C virus NS5b RNA polymerase [48], BACE-1 [42,49,50], MMP-12 [21], HSP70 [51], thrombin [47] and HIV RT [52].

■ Lead characterization & optimization

Once hits have been identified and validated, their interaction characteristics are readily investigated using SPR biosensors [ELINDER M *ET AL.* INHIBITION OF RESISTANT HIV-1 BY MIV-170, A SLOWLY DISSOCIATING NON-NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITOR, MANUSCRIPT SUBMITTED,48,49,51,53–57,GEIT MANN M *ET AL.* KINETIC CHARACTERIZATION OF HCV NS3 INTERACTIONS WITH NS4A AND INHIBITORS. MANUSCRIPT SUBMITTED,58]. The following section describes the basic types of analyses that can be performed in order to get a more-detailed understanding of a lead–target interaction.

Mechanistic analysis

Molecular interactions are typically assumed to be simple and reversible 1:1 interactions:

$$P + L \rightleftharpoons PL$$

without additional molecular species or steps due to secondary effects such as conformational changes or chemical transitions (e.g., as illustrated in **FIGURE 2**). Although this may often be the case, more complex mechanisms are common. The mechanism should consequently be verified for each target and type of compound.

Important basic mechanistic information about an interaction is readily obtained as reversibility, stoichiometry and the number of steps involved in forming a complex are observed directly as deviations from ideal behavior in the data, providing that the experiments are well designed. Mechanistic complexities are, therefore, often already detected when an assay for a target is established, since the sensorgrams are not well described by a simple 1:1 interaction model. They are not as easily recognized when using equilibrium-based interaction assays or indirect assays, such as enzyme-inhibition assays. For example, detection of enzyme-inactivation or time-dependent inhibition with a substrate-based inhibition assay requires a specifically designed experiment. Even more elaborate experimental designs are required to establish the mechanism for time dependence [59]. The researcher must therefore suspect that there is a complexity that should be followed up. Although mechanistic complexities are readily detected with SPR biosensor experiments, it is not trivial to determine the kinetic rate constants for individual steps. For example, the quantitative analysis of tight-binding inhibitors can be challenging if there are no suitable regeneration conditions available (as for the compound in **FIGURE 6**). Experiments may therefore be limited to qualitative analysis using single injections over a sensor surface [ELINDER M *ET AL.* INHIBITION OF RESISTANT HIV-1 BY MIV-170, A SLOWLY DISSOCIATING NON-NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITOR, MANUSCRIPT SUBMITTED,32] or 'single-cycle kinetics' [53]. However, qualitative analysis of an interaction for which the mechanism is known to be complex is often more informative than the blind use of equilibrium-based inhibition parameters (e.g., K_i or IC_{50} values), which can be highly misleading.

Surface plasmon resonance biosensor analysis has, for example, been used to reveal the

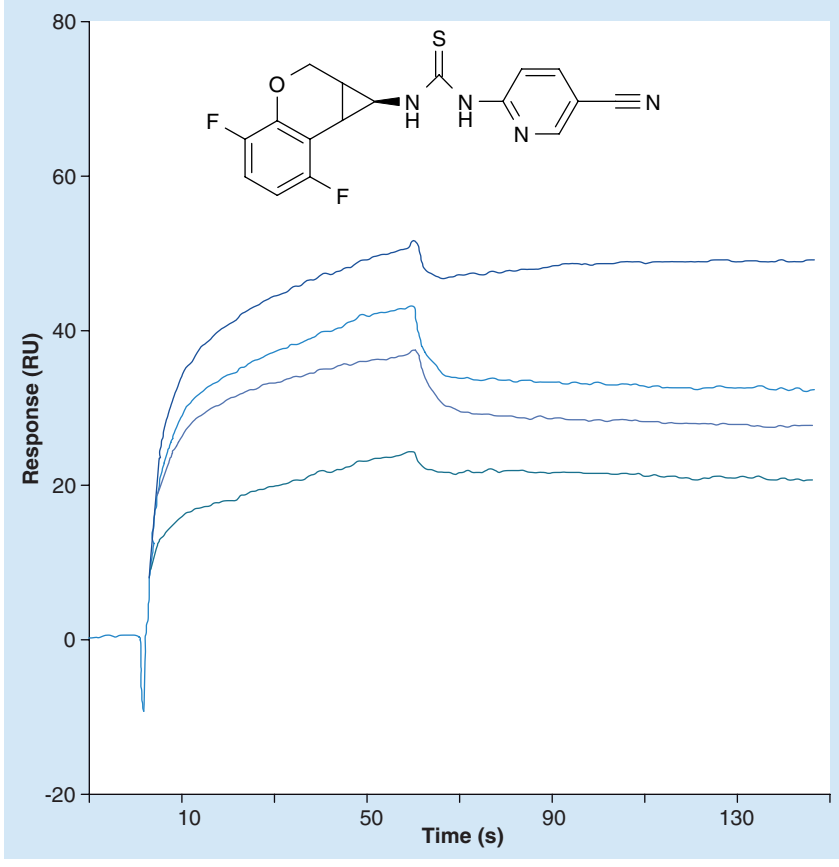

Figure 6. Structure and sensorgrams for a compound identified to dissociate slowly from four variants of HIV reverse transcriptase, screened in parallel using the experimental design illustrated in FIGURE 5.
RU: Refractive units.
Adapted with permission from [32].

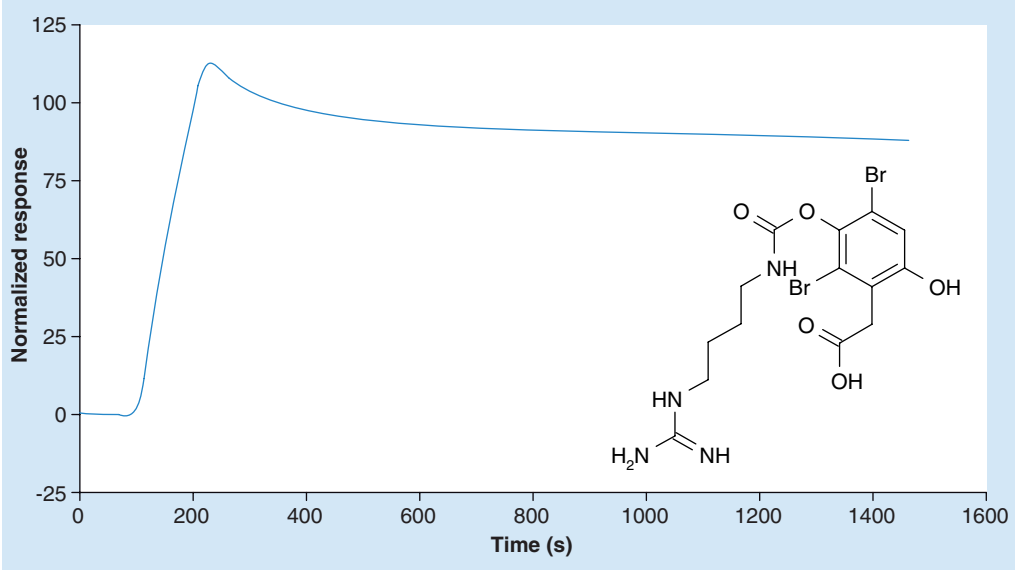

Figure 7. Sensorgram for the interaction between clavatadine A and factor XIa.
Reproduced with permission from [54].

essentially irreversible interactions between hydroxamate inhibitors and matrix metalloproteinase-12, explaining the high affinity for this class of inhibitors [Gossas T *et al.*, Unpublished Data] and the irreversible binding of clavatadine, a natural product inhibitor of the serine protease factor XIa **(Figure 7)**. The underlying mechanism in the latter example was interpreted as involving the formation of a covalent bond between clavatadine and the catalytic serine residue [54].

Similarly, SPR biosensor measurements have demonstrated the complex interaction between cytomegalovirus protease and its substrate [25] and the two-step inhibition of hepatitis C virus NS3 protease by a mechanism-based serine trap inhibitor [Geitmann M *et al.* Kinetic characterization of HCV NS3 interactions with NS4A and inhibitors. Manuscript Submitted]. Mechanistic information for the binding of Ca^{2+} to CRP [29] has also been obtained by SPR biosensor analysis. In this case, it was sufficient to determine the signal at saturation (~10, **Figure 5A**) with that expected for the known stoichiometry for the interaction (~1). The deviation from the expected stoichiometry implies that the known conformational changes upon binding of Ca^{2+} influence the signal noticeably. The data could be used to determine the affinities of the two calcium ions binding to each monomer and, subsequently, to propose a model for which the calcium ion binding sites had the highest affinity for the ion. This study concluded that the function of CRP may be regulated by variations in Ca^{2+} concentrations and is, therefore, of relevance in understanding the role of this protein in disease.

Unexpected complexities in the allosteric interaction between HIV-1 RT and NNRTIs were discovered using a biosensor-based approach. The interaction is generally assumed to be a simple reversible 1:1 interaction, although reports of complexities have been published and some of these have been characterized using more elaborate experimental approaches [60]. The first SPR biosensor study of this enzyme used a series of different experimental designs to revealed four different types of complexities [61]:

- Many NNRTIs bind essentially irreversibly to the wild-type enzyme **(Figure 6)**. There is, as yet, no structural or mechanistic explanation for this very tight interaction (see later), although it does exclude the use of an equilibrium-based analysis for truly evaluating the characteristics of NNRTI interactions with their target;

- The enzyme is present in two free forms, of which only one is capable of binding the inhibitor. By using a drug-resistant variant of the enzyme (from which inhibitors dissociated), it was found that the equilibrium between the two enzyme forms is rate limiting for the binding of inhibitors with rapid

association rates (e.g., efavirenz). This step can be interpreted as involving a relatively slow opening of the allosteric NNRTI-binding pocket;

- The binding of NNRTIs induces a conformational change in the enzyme–inhibitor complex consistent with an allosteric mechanism. It may also be the explanation for the tight binding of many NNRTIs. However, the kinetics of this transition could not be quantified due to the complexity of the model and the limited data that can be obtained for tight-binding compounds;
- Nucleoside reverse transcriptase inhibitors can interact with two forms of the enzyme, distinguished by different kinetics. There was no independent support for this interpretation at the time; however, a later study showed that the enzyme is present in two conformations, primed for binding DNA or RNA, possibly providing an explanation for this heterogeneity [62].

This example illustrates how the mechanistic complexities of an enzyme can be revealed with an SPR biosensor-based approach and that extensive information on leads can be extracted, especially when specifically designed analogues and series of ligands with different characteristics are available [53]. Surprisingly, a more pragmatic approach is often used [55,56].

Kinetic analysis

Kinetic analysis of molecular interactions is clearly the most common application for SPR biosensors. Extensive data analysis using a well-defined mechanistic model and a quantitative determination of kinetic parameters is not always required as sensorgrams provide a very simple and easily interpreted qualitative analysis of the kinetics of interactions. This is often sufficient for comparative studies of lead series or target selectivity. For example, the effect of analyte concentrations and dissociation rates on both complex concentration and stability over time is illustrated in **Figure 8**. This is clearly more informative than equilibrium-based measurements, which simply detect the differences in steady-state levels but do not provide an underlying explanation for the results.

The importance of the slow dissociation of drug–target complexes has recently emerged as a primary kinetic characteristic, of importance for lead optimization [63,64]. Although this is not a new concept as such, being one of the key parameters determined in pharmacokinetic studies, it is now possible to detect compounds with certain kinetic features already at the level of screening, using a biosensor-based approach [32]. Dissociation rates can be expressed as residence time, providing an alternative perspective on the relevance of dissociation rates:

$$\tau = \frac{1}{k_{off}}$$

Since the dissociation rate can be determined during the characterization of hits and leads, it is an attractive parameter to use in the early stages of lead discovery. Despite the obvious advantages of drugs with slow dissociation, there are instances where other parameters are more important, such as in the case of HIV protease inhibitors [65]. Also, if slow dissociation was the ultimate goal for optimization, irreversible inhibitors would be the ideal solution. Although many drugs today are irreversible, it is a characteristic that can cause many side effects and is, therefore, often avoided. It is, therefore, essential to establish the kinetic parameters and other kinetic interaction features that correlate with good *in vivo* efficacy before optimizing a lead on the basis of a single kinetic parameter.

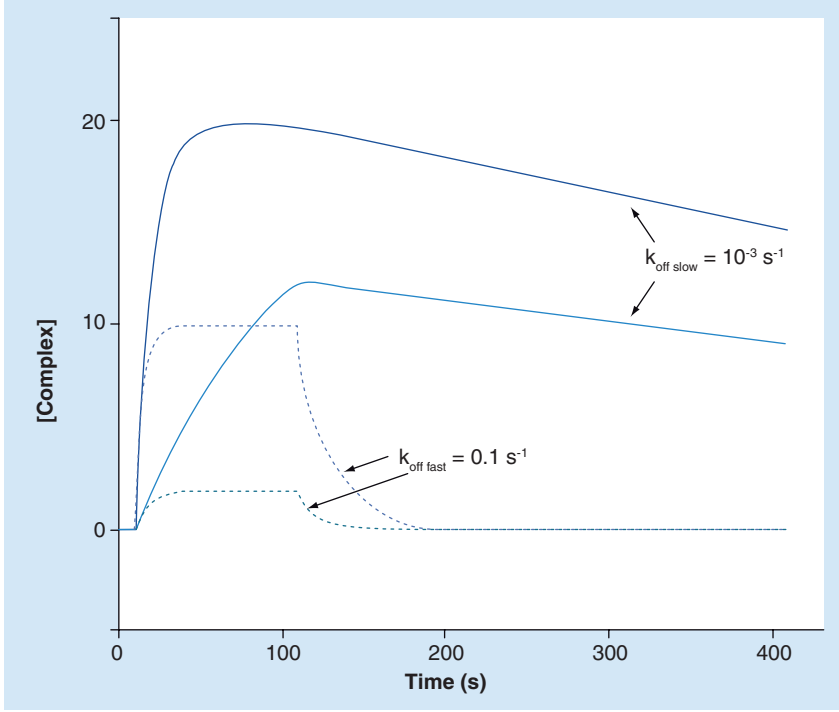

Figure 8. Simulated sensorgrams for interactions with the same k_{on} ($10^6\ M^{-1}s^{-1}$) but different k_{off} for two different analyte concentrations (1000 nM [top] or 100 nM [bottom]) and the same target concentration.
The corresponding affinities can be calculated from: $K_D = k_{off}/k_{on}$.

Furthermore, in light of the complex mechanism of NNRTI inhibition (see previously), it is clear that there are often many kinetic parameters to consider but that only a few can be influenced by ligand design. A full kinetic characterization of interactions with a set of compounds should therefore be performed before dissociation rates alone are used to determine lead optimization.

KINETIC CHARACTERIZATION
Quantitative determination of the rate constants of an interaction mechanism.
A broader definition includes the determination of equilibrium dissociation or association constants (i.e., K_D or K_A)

Structural analysis

Although biosensors do not directly provide structural information, they can do so indirectly. For example, the location of the binding site for a certain ligand can be identified with panels of protein variants (mutants or natural analogues) or with genetically or chemically modified proteins. Competition studies with reference compounds for which the binding site is known are also very useful [42]. A combination of these methods was used to identify fragments interacting with the active site of MMP-12 [21]. Competition studies have also been used to determine if novel NNRTIs of HIV RT interact with the same binding site as the clinical NNRTIs [32,52] and whether there is cross-talk between the different allosteric sites of hepatitis C polymerase [59].

Another aspect of structure that can readily be studied with SPR biosensors is the effect of structural modifications of the ligand or the target on an interaction and the relevance of this in optimization [41,66]. Such studies can generate selectivity/specificity and resistance profiles and suggest structural features that can improve the characteristics of leads with respect to a variety of criteria of relevance for efficacy. In contrast to structure–activity relationship (SAR) studies based on affinities (e.g., K_D) or other equilibrium-based data, structure–kinetic relationships (SKRs) can be performed on the basis of interaction kinetic rate constants [66]. It has been shown that quantitative structure–activity relationships (QSARs) based on affinity may be too crude for reliable results, indicating that it is important to resolve affinity into the individual rate constants, k_{on} and k_{off} for lead optimization [67].

SPECIFICITY/SELECTIVITY
The relative affinity (or other characteristic) of a compound for an alternative target or, conversely, of a target for a set of analogues

STRUCTURE–KINETIC RELATIONSHIPS
The relationship between specific aspects of molecular structure of proteins and ligands and the kinetic rate constants for the interaction. These are typically visualized in k_{on} versus k_{off} plots

QUANTITATIVE STRUCTURE–ACTIVITY RELATIONSHIPS
The relationship between specific aspects of molecular structure of proteins and ligands and defined interaction characteristics, such as affinity. These are parameterized and expressed in quantitative terms

An example of SAR on the level of fragment expansion has recently been performed with thrombin [47]. It used proline as an anchor for binding to the P2 site of the enzyme. By systematically varying the P1 and P3 substituents **(FIGURE 9)**, compounds with detectable affinity and inhibitory effects were identified and their SAR could be elucidated.

In this case, the low affinity of the compounds restricted the analysis to affinities (K_D) since the separate rate constants were too fast for quantification, a typical situation for fragments and low-affinity compounds.

Thermodynamic & chemodynamic analysis

Finally, two additional but very informative types of experiments will be briefly mentioned. These involve measuring interactions under different conditions, either at different temperatures (thermodynamic analysis) or in buffers of different compositions, such as pH, ionic strength or presence of additives (chemodynamic analysis). Such studies can provide important information about the dominating forces and energetics involved in the interaction and are useful for lead optimization. For example, the details of the interactions between inhibitors and HIV-1 protease [68] and hepatitis C polymerase [59] have been obtained by this strategy.

In contrast to calorimetric experiments, which are based on measurements at equilibrium, a biosensor-based approach can also provide the thermodynamic parameters for the association and dissociation of the interaction. However, only a few examples of such applications for small molecules exist [69–72] and there is clearly a need for more research on this topic [73].

Integration with other techniques

As illustrated previously, SPR technology can provide a wealth of information concerning molecular interactions and characteristics of both ligands and targets. The flexible experimental design allows many types of experiments to be performed once an assay has been established. Nevertheless, other assays are also necessary in order to obtain complementary information.

NMR and x-ray methods are essential for determination of the structural details of lead–target complexes. Structural modeling can be

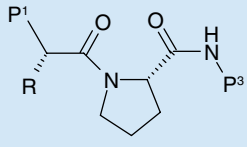

Figure 9. Thrombin inhibitor scaffold studied in a structure–activity relationship analysis using a surface plasmon resonance biosensor approach.
Data taken from [47].

used as an alternative in the initial phases of a project or when experimental methods are out of reach for practical reasons. The need for experimental structural methods can be postponed in a project if SKR analysis can be performed with both target analogues and series of ligands.

Functional *in vitro* assays (e.g., enzyme activity-based inhibition measurements) provide important evidence for the desired functional effect of the compound. The possibility that a ligand binds to the target without eliciting an effect must be excluded. *In vivo* or cell-based assays are also important, in order to confirm that the biophysical and structural parameters used for lead optimization result in physiologically effective compounds. The sooner compounds can be tested and confirmed in such assays, the faster the biosensor assay can be optimized to identify compounds with improved characteristics.

Since the most exhaustive analysis is obtained by combining several of these technologies, it is a matter of resources and the need of each project that defines which methods to use. Ideally, a project has access to the full range of biophysical techniques that can be used for lead discovery. Nonetheless, the order of techniques to be used should be chosen with respect to the assets and drawbacks of the different methods.

Future perspective

The use of SPR biosensors for drug discovery will most likely increase considerably over the next 5–10 years. Like many new techniques, it has had initial problems in becoming generally accepted. This is partly explained by the relatively poor sensitivity of the first instrument models and difficulties in understanding how to design experiments and analyze data in a reliable manner. As a result of new instrument models and more extensive use in both academia and industry, these factors should no longer be a problem and further development of the technique will hopefully increase the ease of use greatly within the next few years. Nevertheless, it will be necessary to train researchers in the fine art of how to perform and interpret experiments. Today, many instruments in academic and industrial laboratories are not being used to their full potential (or at all) due to a lack of experienced users. Much of the pioneering work on new applications is apparently being performed in the pharmaceutical industry, from which little information trickles out and few studies are published (at least in sufficient detail for others to learn from). Few academic groups are yet using the technology for studying the basic and general features of molecular recognition, although they do use it to obtain important information for a certain interaction of interest. The advancement of this technology for lead discovery and optimization therefore relies on researchers in academia and industry collaborating on the deeper scientific and more practical applied aspects. The advent of fragment-based lead discovery has circumvented the need for this type of collaboration somewhat, since academic groups can now venture into the first stages of lead discovery without

Executive summary

Surface plasmon resonance biosensor basics

- Surface plasmon resonance (SPR) biosensors are based on a very sensitive label-free optical detection principle: the sensor surface is typically a hydrophilic and flexible dextran surface with an immobilized target protein.
- Immobilization of sufficient amounts of functional protein is a critical factor for good data.

Sensitivity & information content

- Sensitivity is sufficient for detection of low-molecular-weight analytes and conformational changes.
- The information content is defined by the experimental design and method for data analysis, and material consumption is low.
- Alternative techniques are good complements; however, few other methods can provide the kinetic resolution that time-resolved SPR biosensor experiments can.

Lead discovery

- Fragment-based lead discovery can successfully be performed with SPR biosensor analysis.
- Screening with several hit filters can be performed by SPR biosensors.
- SPR biosensors are suitable for characterization of hits identified by screening of fragment libraries by other techniques.

Lead characterization & optimization

- Mechanistic analysis of interactions provides informative details of interactions.
- Structural information can indirectly be obtained, identifying the binding site of interest.
- Structure–kinetic relationships provide detailed data for lead optimization.

alliances with pharmaceutical industries. This will most likely influence the location of expertise and the scientific stringency of the field of SPR biosensor-guided lead discovery.

Not only are the techniques and the experience in performing and analyzing experiments expected to improve, but the understanding of how to use the data will also increase among researchers not themselves involved in the experimental work. Medicinal chemists, for example, must gain experience in how to exploit the information-rich data for lead optimization. Moreover, it is essential to improve our understanding of how to interpret and use data regarding an interaction (e.g., thermodynamic parameters) that can be obtained but whose relevance for lead discovery is not yet established. SPR is, therefore, an interesting example of a technology that is moving forward the boundaries of science via application-driven research.

Acknowledgements

Thanks to all the researchers whose work has been well documented and published so that others can be inspired and learn how to implement SPR biosensors for drug discovery.

Financial & competing interests disclosure

The author has no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

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