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Review:

Developments in SPR Fragment Screening

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***Introduction*:** Fragment-based approaches have played an increasing role alongside high-throughput screening in drug discovery for 15 years. The label-free biosensor technology based on surface

plasmon resonance (SPR) is now sensitive and informative enough to serve during primary screens and validation steps.

***Areas covered*:** In this review, the authors discuss the role of SPR in fragment screening. After a brief description of the underlying principles of the technique and main device developments, they evaluate the advantages and adaptations of SPR for fragment-based drug discovery. SPR can also be applied to challenging targets such as membrane receptors and enzymes.

***Expert opinion*:** The high-level of immobilization of the protein target and its stability are key points for a relevant screening that can be optimized using oriented immobilized proteins and regenerable sensors. Furthermore, to decrease the rate of false negatives, a selectivity test may be performed in parallel on the main target bearing the binding site mutated or blocked with a low-off-rate ligand. Fragment-based drug design, integrated in a rational workflow led by SPR, will thus have a predominant role for the next wave of drug discovery which could be greatly enhanced by new improvements in SPR devices.

Keywords: fragment-based drug design, surface plasmon resonance, fragment screening, molecular interaction.

1. Introduction

Since its beginning 15 years ago, the fragment-based drug design approach (FBDD) has played an important role in drug discovery by biotechnology and pharmaceutical companies (1,2). Nowadays, this strategy has generated more than 30 fragment-derived compounds in the clinic development and at least one drug on the market : Zelboraf (vemurafenib) (3). The FBDD approach uses screening libraries composed by 500 to 3000 low complexity molecules with typical molecular weights (MW) in the 100–300 Da range and cLogP<3 [2]. FBDD has several key advantages compared to a classical

High-throughput screens (HTSs) of up to 1,000,000 compounds (MW=250-600 Da, cLogP <5). Despite being thought of as weak in potency in term of affinity, fragments are actually efficient binders as they can form high-quality interactions (4). Their low structural complexity decreases the probability of interaction mismatches with the protein target but can nevertheless lead to hits, albeit with low affinity (0.1-10 mM). However, the low molecular weight and reduced cLopP of initial hit fragments are of positive impact during the optimization process. This optimization, generally associated with structural biology studies to determine the fragment-protein complex, is achieved by growing and modifying the initial fragment to a proximal binding site, or by linking two different fragments together. The efficiency of the molecular optimization can be monitored using several metrics like the Ligand efficiency (L.E.) value which is the free energy of binding divided by the number of heavy atoms (5). Moreover, with small size libraries, the combination of fragments leads to a better coverage of the 'chemical space' than drug-like compounds and allows investigation of non-conventional classes of drug-targets (4). As FBDD was adopted by academic groups, small biotech and large pharmaceutical industries, several commercial companies (Maybridge, ChemBridge, Asinex…) have assembled fragment collections which may, or may not, have been selected based on the 'Rule of Three' in which molecular weight is <300, the number of hydrogen bond donors/acceptors is each $\leq$3 and cLogP is $\leq$3 (6).

Whatever the physicochemical filters used for a well-designed library, an important criterion for success is a high fragment solubility in aqueous buffer. Whereas classical compounds are tested around 10 µM in a standard HTS, fragments have to be used up to millimolar concentrations adapted to the expected low affinity for their targets. An 'oriented' library could be designed to the target specifications (enzymes, membrane receptors, protein-protein interactions) and to the screening method used (NMR, X-Ray crystallography, SPR…) (7).

The low affinity of fragment hits necessitates high sensitivity biophysical detection techniques. These techniques fall into two types: those that are conducted in solution such as NMR, X-ray crystallography, calorimetry, thermal shift and mass spectrometry and heterogeneous other techniques

with immobilized protein targets such as Surface Plasmon Resonance (SPR) (8), interferometry (9), FujiFilm-SPR (10) and the SensíQ Pioneer-SPR (11).

SPR is a rapid and informative method that gives access in real-time about binding constants and stoichiometry for low-, intermediate-, and high-affinity interaction partners such as fragments (K_D; 10^{-6}-10^{-3} M), compounds (K_D; 10^{-8}-10^{-4} M) and antibodies (K_D; 10^{-12}-10^{-7} M), respectively (12). With the technical improvements in the last ten years, SPR devices are sufficiently sensitive and well-configured to be suitable for rapid medium-throughput screening of fragments (13,14). The SPR approach are now well validated on different classes of targets such as proteases (15,16), kinases (17), GPCRs (18,19) and protein-protein interactions (PPIs) (20–22). In recent years SPR has become an important screening method to confirm, prioritize, characterize and more recently identify fragments (23). This review will focus on the development of SPR in fragment screening, after a brief introduction on the principles of SPR, the evolution of the devices in the drug discovery field. The strengths and adaptations of SPR are described in the context of other techniques. We analyze new applications of SPR for challenging targets such as GPCRs, protein-protein interactions (PPI) and enzymes. Finally, in the "Expert Opinion" section, we discuss the conformational stability of the immobilized protein target and the selectivity assay as key points for a relevant FBDD screening. Then, we propose an integrated work-flow based on SPR.

2. Surface Plasmon Resonance

SPR is a label-free biophysical method that determines, kinetic constants, affinities and stoichiometry of a molecular interaction in real-time (8,24). Briefly in the Kretschmann configuration (**Figure 1)**, the physical phenomenon of SPR is based on a metal surface (usually silver or gold) separating 2 dielectric compartments, one is a prism with higher refractive index (RI) and the second with lower refractive index can be a liquid or the air (25). The prism side of the surface is illuminated by a polarized and monochromatic incident light, in total internal reflection conditions, which generates an evanescent wave. In the liquid compartment, any localized change of the mass due to the capture of soluble molecules by the immobilized target induces an RI modification which leads to a variation of the critical angle of the reflected light, at a minimum of intensity, called resonant angle. Accordingly,

the real-time SPR measure is given by an arbitrary resonance unit corresponding to a change of resonant angle subsequent to a change of surface density (1RU=0.01 mDegree = 1 pg of protein per mm^2). The SPR procedure thus involves first, the immobilization of one interactant onto the gold surface of a sensor chip, then the other partner is injected into the flow cell over the coated surface during the association phase. The replacement of injected sample by the flow buffer allows the dissociation phase to be studied. This real-time monitoring provides access to kinetic association (ka) and dissociation (kd) rate constants, affinities at equilibrium (K_D=kd/ka) and the stoichiometry of the interaction.

3. Evolution of SPR devices in FBDD

The first Biacore SPR devices were created by Pharmacia in 1990 and they were dedicated to studying protein-protein interactions. Nowadays, about fifteen manufacturers offer SPR devices (26). Since 2005, SPR improvements include optimized sensitivity, higher throughput and easy-to-use software. These new apparatus are suitable for drug applications and also to detect weak interactions (K_D in mM range) as required for fragment evaluations.

Two machines from GE Healthcare are the most used for fragment approach. The high-cost A100 device with five sensor spots, each with 4 channels, was recently upgraded and released as the Bia4000 model. Because of its high cost, the Bia4000 is mostly intended for pharmaceutical companies. The Biacore T200 has just one sensor with 4 channels and is more widely used by academics or start-ups. Recently, a new configuration of the T200, called the S200, which is uniquely dedicated to drug and fragment discovery was launched onto the market. Also, SPR imaging, made of a coupling between a SPR Kretschmann system and a camera CCD with an enhanced throughput, can be used in a FBDD approach (27).

During the FBLD 2014 conference in Basel, SensiQ presented a new SPR instrument, the Pioneer FE (Fragment Edition), which was co-developed by scientists at both Genentech and GlaxoSmithKline. SensiQ is designed to address screening and validation of hits using 2 types of dynamic injection OneStep™ and FastStep (11) or diSPR® (28) and active selection software (29). Briefly, the OneStep

injection consists of a wide concentration gradient (3-4 orders of magnitude) of the analyte formed by diffusion in a coil-capillary tube before reaching the detection cell, which allows a dose-response in one single sensorgram and a resulting robust K_D evaluation. This new device has been validated in a fragment screening comparatively to the S51 from GE Healthcare in an application note (30). With FastStep, the gradient of *in-situ* dilutions is obtained by an instant mix during the injection. The proof of concept for primary fragment screening was reported by Myszka and coworkers from a library of 320 compounds against the well-characterized Carbonic anhydrase II which is known for its stability (11). In the near future, SPR microscopy (31) and an emerging SPR based on nanostructures (32) which propose a higher throughput and sensitivity, could also stimulate the field.

4. Strengths and limitations of SPR for FBDD

The predominant SPR experimental protocol involves the direct binding of fragments on an immobilized target, which is usually a pure and active protein (**Figure 2A)**. However the reverse scheme, where chemical fragments are immobilized on a microarray is available from NovAliX (27). Protein immobilization can be achieved using covalent coupling via -NH2, -SH, -CHO, -OH, or -COOH groups and non-covalent coupling strategies with affinity tags such His-Tag or the couple streptavidin/biotin. The most commonly used method is covalent coupling of target on a dextran chip. The non-reticulated dextran polymer forms a flexible 3D layer with a higher loading capacity than a 2D self-assembled monolayer. For biotin immobilization, either a biotinylated spacer arm can be chemically added to a primary amine group in proteins or a mono-biotinylated AviTag can be introduced at the N- or at the C-terminus of the protein depending on the tag's interference with protein activity or expression (23). Interestingly with such AviTag, a Biotin-capture target on tetravalent streptavidin (SA chip) can conduct to a well-oriented and high-loaded protein. A new regenerable sensor biotin-capture kit is now available from GE Healthcare and was recently used for FBDD (21,33). The possibility to periodically re-immobilize the target decreases the probability of denaturation during the screening campaign and ensures a high reproducibility in the results. Also, capture of proteins can occur via a HiS-8/10 tag protein on a NTA sensor chip (34).

4.1 Low consumption, rapidity and high sensitivity

The immobilization of the target on a 1.2mm^2 channel surface allows much less material to be used than for other methods (23). As reported by Giannetti, less than 1mg of protein would be sufficient to perform a total fragment screening assay and the associated structure-activity relationships study. The relative low multiplexing (4 channels or 20 spots) of currently used SPR-devices compared to other techniques, like high throughput NMR for instance, is largely compensated by the rapidity of the SPR. Indeed several hundred cycles of association and dissociation of a single fragment, followed by very short washes, can be performed over a 12h period on a Biacore4000.

Several parameters for the sensitivity of a fragment binding response have to be considered. First, as the change of surface density with the binding of one fragment is low, a high level of immobilized active protein is needed. Then, a stability of the baseline and a low noise level are crucial parameters during the screen of a fragment. In good conditions on a Biacore T200, a significant signal of around 1 RU can be obtained. However, an excessive level of immobilized target can lead to protein aggregations on the chip (crowding effect) and a reduced binding site accessibility. Mass transport limitations and rebinding are common consequences of high level of target immobilization. However, these effects are reduced or even canceled due to the low molecular weight of fragments and their fast dissociation from the target (23). The evaluation of control ligands, when possible, at different levels of immobilized protein is very informative on the quality of the test as well as on the stability of the protein in time.

Due to their putative low affinity in the millimolar range, higher fragment concentrations, up to 5-10 fold the KD, should be used in SPR screening conditions. However, such concentrations are rarely reached. Fragments are usually solubilized in DMSO, before the final dilution in aqueous buffer. DMSO presents a high refractive index that may impair the sensitivity of the measure. A compromise is necessary between concentration of fragments and % of DMSO suitable for a SPR run and usually, the screening is performed at around 5% of DMSO. A correction of the responses by a prerequisite DMSO calibration must therefore be performed and this has been automated in the latest generation of software. An exhaustive view on how to prepare buffer and compound for a fragment screening is presented in the review written by Anthony Giannetti (23).

4.2 Discriminative data and false-positive detection

4.2.1 Selectivity

Using a multi-surface sensor, SPR allows differential scanning assays against several proteins or control conditions which is very useful for studying the binding selectivity and for detection and removal of false positive binders. In a typical experiment in addition to the protein target, one of the 4 channels of a sensor is assigned to a blank flow-cell to subtract the background noise, another flow cell is dedicated to an off-target protein (e.g. of nearly the same MW) or with the inactive form of the target owing an unstructured or blocked binding site. It can also often be very useful to immobilize a closely related family-member of the protein target to probe the specificity more precisely. On multiplexed SPR-devices like Bia4000 up to 4 different proteins can be tested in parallel with all the controls included (14,35). The rationale of the selectivity/specificity studies is discussed in the 'expert opinion section' below.

4.2.2 Equilibrium or kinetic analysis and false-positive removal

Generally with fragments, kinetic constants cannot be obtained from sensorgram analysis because the association and dissociation phases are too fast. Habitually, fragments are analyzed at one concentration to obtain the ranking of their binding values and with increasing concentrations to measure affinity and stoichiometry values (**Figure 2A**). The K_D is evaluated by a steady-state fitting model from dose response curves. However, the low solubility of compound frequently impedes the K_D determination as high enough concentrations cannot be reached. After the noise/off-target double-blank and DMSO corrections, these normally box-shaped sensorgrams inform on the binding level (in RU) and allow to detect inconsistencies like over-stoichiometry, irregular curves profiles, non-dissociating compounds or carry-over effects (36). The detection of hyper-stoichiometric responses, superior to 5 times (SR>5) the 1:1 Langmuir adsorption model, strongly implicates a fragment as a false positive binder (23,37). The stability of the coated–target must be periodically controlled with positive and negative compounds and the reproducibility tested by measurement of replicates. The assay quality is evaluated by a statistical Z' factor (35,38).

Recently, several protocols have been reported to confirm the interaction or the nature of the binding site of a fragment. As shown on **Figure 2B,** a competition assay with a control ligand, another drug or if it is not possible another positive fragment, can be included in the screen flow.

In an alternative protocol, reported by Giannetti et al., called 'simultaneous competition assays', fragments are tested in direct binding ($\mathbf{R_{Fn}}$) and secondly, or in parallel on a second apparatus, with the competitor ($\mathbf{R_{Fn\ +cb}}$) continuously present in the buffer (**Figure 2C**). All the responses are further treated to select a strongly putative competitive candidate compound based on the S factor which is defined as $\mathbf{S=(R_{Fn}-R_{Fn+Cb})/(R_{Fn}+R_{Fn+Cb})}$ (29) (**Figure 2C**). Fragments with S>0.3 are considered as competitive binders (39) whereas non-competitive binders have S values closer to 1 and uncompetitive binders have an S>1. Non-competitive and uncompetitive binders require further confirmation and validation by orthogonal techniques.

A few reports confirmed hits using a SPR reversed protocol, i.e. by injection of a mix of target and fragment, in parallel to injection of the target alone, over an immobilized known ligand, as shown in **Figure 2D** (21) (40)**.**

5. Comparison of SPR with NMR and X-Ray crystallography

SPR, NMR and X-ray crystallography are the major biophysical techniques used for FBDD (41). Their relative virtues for fragment discovery, i.e. the SPR properties discussed above and the major characteristics of NMR (42,43) and X-ray crystallography methods (44) are compared in **Table 1**. Several technologies such as Differential Scanning Fluorimetry, Microscale Thermophoresis are emerging for fragment Screening (see (1) for review). NMR, was the first technique to be used for fragment screening it can be performed in solution, either on the ligand, by monitoring the ^{1}H- (STD, (45) and waterLOGSY (46)) or ^{19}F-spins of the fragments, or from the detection of signals from ^{15}N- or ^{13}C-labeled target proteins (47). We also have to mention another NMR approach, called TINS, using immobilized protein but not reported in Table1 [45]. NMR has several advantages which maintain its relevance. For example NMR can give structurally relevant data for protein with MW lower than 30 kDa whereas label-free NMR on ligand is easily adapted to higher throughput with fragment mixtures. Although NMR is invariably more product- and time-consuming than SPR it can

nevertheless be used in direct binding and in competition assays with another ligand to validate hits or for secondary screening (48).

X-ray crystallography is widely used in hit validation as it can be performed on complexes formed by soaking a crystal of the protein in a solution containing a high concentration of fragment, or by co-crystallization of the protein and a fragment. Crystallography is more time-consuming than the other techniques and notoriously difficult to adapt to high-throughput, even if recent progress has been made in parallel and automatized devices (49). Crystallization is the major approach to provide crucial structural information for lead optimization although it remains impossible for many targets including most membrane proteins.

For SPR in a FBDD approach, the main limitation results to the high level of immobilized targets as discussed in the expert opinion section.

6. Remarkable applications and developments of SPR

Several developments of SPR were recently reported or adapted for FBDD screenings on important and challenging therapeutic targets (50,51). These improvements concern the enhanced stability of the immobilized targets, the characterization of the nature of orthosteric or allosteric binding sites and the correlation with orthogonal biophysical techniques. Based on classes of targets, the next section illustrates these different aspects through a selection of recent reports.

6.1 GPCR

In the field of transmembrane receptors, G-protein coupled receptors (GPCRs) are one of the most important therapeutic classes, as they concern about 25% of authorized drugs. GPCRs constitute a large family of around 800 proteins with high sequence similarity, especially in their orthosteric binding sites. GPCRs have been difficult to screen because of purification problems including low expression and low stability. Until recently, HTS were based on overexpressing receptor cellular screening. One way to maintain the functionality of the receptor during and after the immobilization is to engineer thermostabilized receptors via a number of point mutations. This strategy was first reported for the adenosine A_{2A} receptor and later on the [illegible]receptor with identification of fragments

with K_D in the 10 µM range (34,52). However, thermostabilized mutated GPCRs lack some pharmacology properties as they are stabilized in either the agonist or the antagonist bound conformation (53). In the last 3 years, the group of Navratilova has reported SPR screenings on wild-type GPCRs using, first a library of ligands (54), and then a library of fragments (18,19). Aristolelous et al. described a screen on the native sequence of the human β2 receptor bearing a C-terminal FLAG tag and an N-terminal histidine 10 tag to capture it on an NTA sensor chip. The activity of the immobilized receptors was confirmed by SPR with the binding of agonist (fenoterol) and antagonist (alprenolol). In the reference channel, the control protein was the receptor itself with the binding site blocked with a slow-off-rate agonist compound. With this protocol and a library of 656 fragments, 5 fragments were validated by dose-response assays with K_D from 17nM to 22µM.

Employing a parallel screen on an immobilized target**,** either in the presence, or not, of a low-off-rate ligand occupying the orthosteric site, conducts to a better understanding of the binding nature of fragment hits. Also, this approach has the added advantage of simultaneously screening for allosteric binders. Proof of concept was provided by Navratilova et al., with molecules of molecular weight slightly higher than fragments, on receptor CCR5 blocked by a strong ligand, Mariviroc (K_D 25nM). Two previously known allosteric compounds were included in the screen to validate this protocol (54).

6.2 Non-enzymatic proteins

Nicotinic acetylcholine receptors (nAChRs) are pentameric ligand-gated ion channels, or Cys-loop receptors, involved in the fast synaptic signaling. Cys-loop receptors are therapeutically targeted by several classes of drugs for central and peripheral nervous system diseases (55). In a recent article, Spurny et al used a SPR fragment screening method to identify novel allosteric binding sites on a α7 nicotinic receptor chimera, called α7-AChBP, constructed from the human α7 ligand-binding domain of the nAChR and acetylcholine binding protein (AChBP) (56). This chimeric receptor has a high sequence similarity to the native nAChR, especially in the surface-exposed pockets and loops. In the aim of discovering potential allosteric fragments, the screen was carried on α7-AChBP and *Capitella* AChBP as control proteins using two different strategies applied in parallel. The first strategy was to block the orthosteric site by preincubation with a high-affinity and slowly dissociating

ligand (-Bungarotoxin) and the second strategy was to use a cross-competition assay (**Figure 2B**) with *d*-tubocuranine as this ligand has a fast dissociation rate and thus could be used as competitor and positive control. From a library of 3000 fragments and a single concentration screen, 302 potential allosteric fragments were identified and 24 were confirmed by dose effect, competition and selectivity. 5 allosteric hits were co-crystallized with -AChBP blocked by an orthosteric ligand (lobeline). In combination with electrophysiological experiments, structural study allowed to identify 3 allosteric sites in the -extracellular domain: a novel one called the 'top-pocket' which is surface-exposed, a second site called the 'vestibule pocket' corresponding to an allosteric site of an -nAChR bacterial homolog and the last site close to the agonist sub-pocket which corresponds to a binding site for the anesthetic ketamine in another bacterial homolog of the receptor.

6.3 Enzymatic proteins: Kinases and metalloproteases enzymes

6.3.1 Serine/threonine kinase

In 2011, Pollack et al. reported a strategy to evaluate a fragment-based drug discovery approach using the BioFocus library to find interactors of the P38 kinase in such a way as to select for allosteric compounds and specific binders (17). The BioFocus library was generated according to the Rule-of-Three and was not designed to be used against a particular class of target. A set of 266 fragments (200 µM) was screened for binding by SPR to active p38 selected 24 hits were higher than 50% of the theoretical Rmax (K_D from 0.2 to >10 mM). A study of these fragments by cross-competition (**Figure 2B**), was performed with a known ATP-binding site inhibitor (SB203580) at saturating concentration. 4 fragments gave additive-responses demonstrating a non-competitive behavior and 8 were found to be competitive binders. These 12 fragments were investigated by X ray crystallization studies and three were found to bind within the ATP-binding site whereas the binding site of one fragment was a potentially allosteric distal ATP site, close to the C-terminal MAP kinase insert region. Moreover, a relatively good correlation was observed between the SPR results and a binding screen based on a mobility shift assay.

More recently, Grädler et al. published a similar approach to identify compounds that compete with ATP, on focal adhesion kinase, using a library of 1920 fragments tested at 2mM (57). At this high

concentration about 80% of fragments were positive so, to discriminate true binders, positive fragments were tested again in presence of a high-affinity ATP-competitive binder (PF-562,271). 180 fragments bound less in the presence of inhibitor and of these 105 hits had K_D from 13 µM to 3.5 mM. Finally, 50 compounds with LE. from 0.21 to 0.61 were investigated for their mode of binding by soaking and co-crystallization experiments which led to 41 structures of the kinase with fragments within the ATP-Site.

6.3.2 Metalloproteases

The metalloproteases (MMPs) are a family of 20 enzymes that are closely related in terms of structures and functions, all of which have a catalytic structural zinc ion. Nordström et al. proposed the identification of MMP12 inhibitors by a selective SPR-fragment screening of 245 compounds from a partially focused library (58). To specifically target the active site of MMP12, the screening was performed by SPR in parallel against Carboxic Anhydrase II (CA) which has a similar catalytic site. The main difficulty resulted from the instability of the MMP12 which is susceptible to auto-proteolysis which caused a drift of the baseline during the experiment and an increased consumption of chips. To solve this problem, two variants of inactivated MMP12 were immobilized on a BIA4000 sensor chip in parallel with MMP12 WT and CA as control off-target. The inactivation of the enzyme was ensured either by a mutation or by adding an inhibitor during the immobilization. The screening strategy was double: by direct injection of all the fragments alone at 200µM (**Figure 2A**) and by a competition assay in presence of a ligand (ilomastat) which blocks the active site (**Figure 2B**). In this assay the authors were not interested in allosteric binders. 8 binders of the MMP12 WT and 16 binders of stabilized MMP12 were retrieved from a semi-oriented library of 290 compounds. After a dose-response assay by SPR (**Figure 2A**), 5 hits were selected and confirmed in steady-state activity assays by inhibition of the enzyme. Comparatively to 75% inhibition at 2.5 nM of ilomastat, one fragment caused 100% of inhibition (IC50 value of 0.29 µM), another 60% and the other three less than 30%. It was possible to follow the proteolysis by simple observation of the baseline drift.

In 2010, Boettcher et al. reported an FBDD screen of 2 proteases, the trypsin serine protease and the MMP12 metalloprotease (59). For each target, a set of 352 fragments related to already known “NMR

positives" and "NMR negative" binders to these targets were assembled. The aim was to compare hits obtained by several biochemical techniques (enzyme activity-based fluorescence intensity, fluorescence lifetime, and mobility shift assays) as well as by NMR and SPR biophysical techniques. For the SPR screening approach, the trypsin and MMP12 were immobilized respectively to 6000 and 8000 RU on a ProteOn XPR36 Chip (Bio-Rad, Hercules, CA) with an estimated percentage of active immobilized protein at 50% and 70%, respectively. This point is further discussed in the 'Expert Opinion' section below. The conclusion of this study was the SPR based assays and the 3 biochemical assays were comparable in terms of false-negative and false-positive rates. The majority of false negatives in SPR and biochemical assays have a K_D (determined by RMN reporter assays) above 1 mM. Interestingly, the false-negative rate was relatively high in the case of the trypsin and authors have concluded that it could be related to the lower percentage of active immobilized protein.

6.4 PPI

Protein-protein interactions (PPIs) with about 130,000 binary possibilities in the human proteome represent a huge target-space to identify new drugs that act on cell signaling pathways (60). The development of inhibitors of PPIs is attractive but remains more challenging than traditional drug discovery as their druggability is limited due to large and rather flat interfaces (61,62). However, in the last decade several PPI inhibitors were developed based on a FBDD approach (63) including an inhibitor of BCL2, called Navitoclax, which is currently in clinical trial. PPI inhibitors can be orthosteric or allosteric disrupters as well as, more rarely, stabilizers. The orthosteric binders target the interface and preferentially some key residues called "hot spots" (64) whereas the allosteric ones modify the affinity of the two partners via an interaction with distal sites to the interface (60). There are few publications on PPI inhibitors from a FBDD using SPR. Most of these publications used SPR as a validation step after a primary screening performed by NMR or X-Ray crystallography against one partner of the interaction (65).

Recently, Rouhana et al. reported the identification of fragments interacting within small pockets around hot spots on the Sec7 surface of the nucleotide exchange factor (GEF) Arno. This GEF activates the GDP/GTP nucleotide exchange of the small G protein Arf1 (21). From a library of 3000

compounds, thirty-three fragments were selected from virtual docking on the surface of ARNO and tested in a fluorescence assay. The four positive fragments were then evaluated for direct binding by SPR to an AviTag-Arno Sec7 domain immobilized on a CAP sensor. The negative control reference was the adhesion protein Δ-Bd37, from Babesia, immobilized by biotin capture. Finally confirmed fragments were investigated by an Inhibition assays using the protein Arf1 as immobilized ligand (**Figure 2D**).

After confirmation of the interaction to ARNO by NMR, the binding mode of 2 fragments, with K_D in the mM range, were solved by X-ray crystallography. An interesting point in this study comes from the virtual screening used to select the fragments as the mode of binding of active fragments were similar in the docking pose and the 3D structure which suggests that computational techniques can be helpful, even on challenging targets such as PPIs.

7. Conclusion

Recent publications, from academic or pharmaceutic groups, have reported the use of SPR on challenging targets as a technique to validate fragments and more recently as a primary screening method. This heterogeneous approach is highly reproducible and its quality can be verified by statistical tools. The number of articles about SPR certainly underestimates its impact in FBDD as many research programs are developed by biotechnological societies and pharmaceutical industries who prefer secrecy to publication (**Figure 3**). However, several blogs and recent books confirm the important of SPR in the future of drug-discovery (1,29,66).

8. Expert opinion

8.1 Conformational stability during and after immobilization

In SPR, one of the main concerns is the immobilization and conformation stability of the target. Although the immobilization on SPR-surfaces allows a marked reduction of protein consumption compared to techniques that treat proteins in solution, the covalent link at a high density, which is a *sine qua non* condition for a fragment screening by SPR, may lead to partial inactivation effects,

crowding and local aggregations. A recurring reproach was random protein immobilizations to change affinity parameters by flexibility lost, especially when covalent link is done by an uncontrolled numbers of point attachments. With good practice and a relevant experimental protocol, this criticism has often been invalid (67). However, in case of FBDD, additional recommendations can be suggested. First, an optimized presentation of the target by using oriented tags at the C or N-terminus should lead to an improved active conformation. Thus, reducing deleterious effects, oriented protein may help to decrease the level of immobilized target necessary to obtain sufficient RU responses. Second, during the coupling step a reversible inhibitor can be used to protect the active site. Then, for low stability targets like GPCR and certain PPIs, regenerable sensor chips are a prerequisite, even though this approach may require more protein. Every target is a special case and different immobilization strategies must be tested and compared before starting the screening process, however, the AviTag appears to be an efficient solution (21,23).

To control the target integrity several ligands should be periodically inserted during the fragment screening as variations obtained with positive binders as well as a baseline drift inform about the stability and the reproducibility. Some software packages using cubic polynomial regression or locally weight regression (LOESS) propose a normalization of the responses according to these fluctuant parameters, which increases confidence (29).

8.2 Selectivity can increase false–negative fragments

SPR is suitable to screen against different immobilized targets and thus allows the study of binding selectivity or specificity of a fragment hit and also to filter a library from promiscuous aggregators (36). Selectivity investigation, if widely recommended by the community in numerous reports, may be questioned as illustrated in the publication of Wielens et al. (68). They comparatively screened 500 fragments against the HIV-1 integrase domain by NMR and SPR, using an ‘off-target’ to investigate the selectivity, and resulting hits were analyzed by X-ray crystallography. They observed that several SPR fragments, which bound on the off-target protein and thus had been removed from the study as non-specific fragments, were nevertheless confirmed binders by NMR and X-ray crystallography. In this context, for both orthosteric and allosteric binding sites, an alternative strategy is to focus

the test of selectivity of a protein by mutating the active site or blocking with a low-off-rate ligand, as discussed above. The matter of selectivity/specificity could then be treated later in the lead optimization.

8.3 A FBDD work-flow focused on SPR technology

For FBDD, the necessity of using several orthogonal techniques, mainly NMR and X-ray crystallography and SPR is not a matter of debate. An integrated work-flow has become standard to decrease artifacts, false positives and negatives as well as to validate fragment hits. Recent advances in SPR methods and devices: that it is less product- and time-consuming, the highly informative data that it yields and its capacity to be used on challenging targets such GPCRs, ensure that SPR will have a predominant role in FBDD and drug-discovery in the foreseeable future. The **Figure 4** summarizes the rational of a strategic work-flow lead by SPR as the primary screening method. The knowledge of the target in terms of stability, functional interactions, known ligands and mechanisms of regulation, inform rational strategies for immobilization, selection of the set of control proteins and fragment screening conditions. SPR can contribute to this knowledge and to evaluate the efficiency and the sensitivity of the implemented screening protocol.

Further developments in FBDD, with the elaboration of more potent and eventually target- focused libraries that could be designed by computational docking techniques (21,69,70) or by deconstruction of high-affinity ligands (71) will likely save time and increase the hit rate in the near future. Then, a simple removal of residual sticky fragment binders by a rapid SPR pre-clean screening (without control binders, reference subtraction and DMSO calibration) could be effective before the primary screening (**Figure 4**). The SPR primary screening could then include 2 steps combined in one. First, the binding analysis at one concentration or at multiple concentrations by a dose-effect measurement. Then, selected hits (RU levels, K_D) can be confirmed in a second screening by simultaneous competition screening with the competitor in the buffer or by cross competition as schematized in **Figure 2C**. These screens will also be simplified, in terms of time and product consumption, by the use of new injection devices such as the SensiQ® Pioneer. In certain cases, fragment hits may also be confirmed using the reverse protocol called inhibition with an immobilized ligand. At this stage, a

ranking of fragments, stoichiometry of the interaction and details of orthosteric/allosteric nature of the binding site could be obtained.

Finally, the NMR and X-ray crystallography orthogonal techniques could be introduced in the work-flow to validate and select fragments for the subsequent molecular optimization steps. NMR is a sensitive method for detection of ligand binding sites and for hit validation (72). X-ray crystallization of the hit-target complex leads to selection of the most promising candidates for fragment optimization and growth. Not all proteins are accessible for crystallography study including membrane-proteins which are often problematic, and here too SPR screening can help by giving precious information to input into further soaking or co-crystallization protocols.

Financial and Competing Interests Disclosure

The authors have 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.

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Figure Legends:

Figure 1 : Kretschmann system of SPR: Optical system of total reflective light coupled with the gold sensor surface of the chip. Near the surface, each variation of concentration leads to a variation of refractive index of the liquid media (**RI_{liquid}**) and a proportional variation of the resonance angle (□). The measure in real time of the angle variation (A) is converted in RU in function of time to give the sensorgram (B). □= 0,1 mdegre = 1 Resonance Unit (RU)

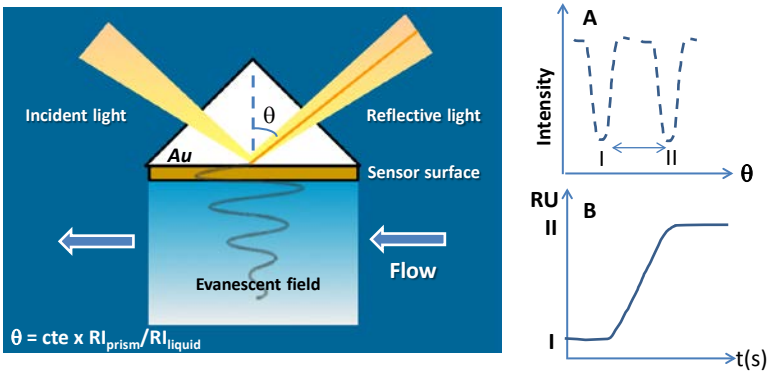
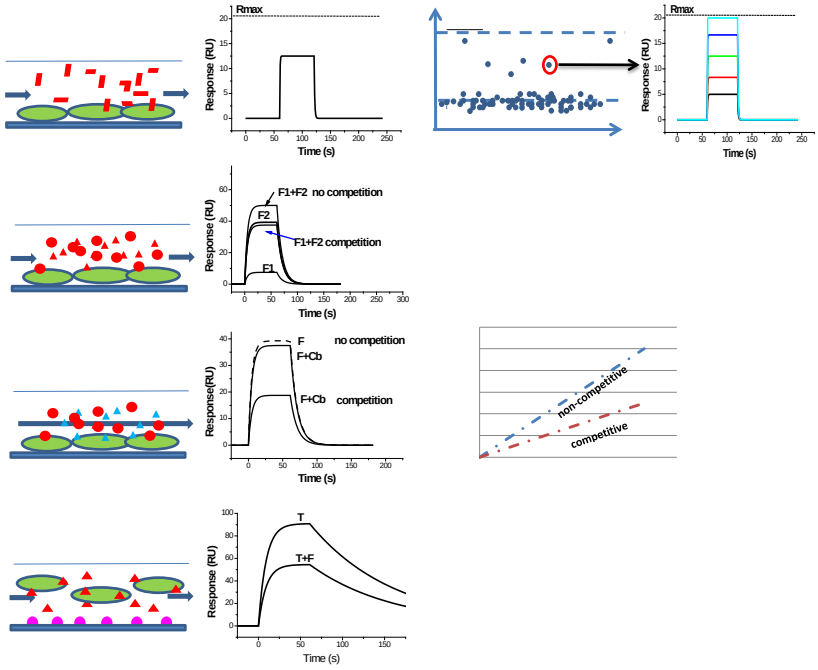


Figure 2. SPR strategies in FBDD. Scheme of protocols and typical responses.On the left is a diagram showing four different types of binding. On the right appear charts of explanation. Directly to the right of each cartoon is a sensorgram from a typical SPR device showing the readout for each type of binding (A-D)



(A) Direct-binding:

- Sensorgram of one fragment injected at a single concentration on an immobilized target,

- RU-ranking of n fragments each injected at a single concentration,

- Validation by dose-response of selected fragment.

(B) Cross-competition:

- Comparative responses of F1, F2 and (F1+F2).

(C) Simultaneous competition:

- Comparative responses for F1 alone and in presence of a competitor continuously in the buffer (F+Cb).

- Graph of a competition diagram (LOESS approach) showing the area delimited by S=0,3 for competitive and non-competitive fragments discrimination.

(D) Inhibition assays:

- Comparative responses of T and (T + F) injected on immobilized ligand.

F: Fragment (red); T: protein Target (green); L: Ligand (magenta); Cb: Competitor in the buffer (blue)

Figure 3. Bibliography study from Web of Science . (A) Published articles per year containing in the title and topic “fragment AND screening NOT antibody” then refined by topic “SPR OR surface plasmon resonance OR Biacore” (a total of 120 from all Databases after removal of irrelevant articles)

and (B) Number of citations per year (sum of the times cited: 2174; sum of the times cited without self-citations: 1853). This report reflects citations to source items indexed within All Databases.

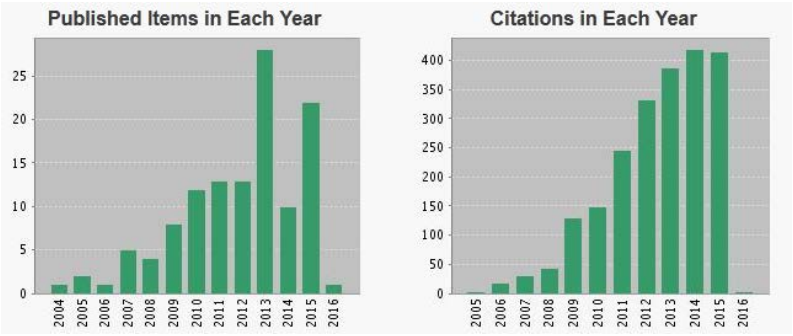


Figure 4. FBDD screening workflow leads by SPR approach

SPR screening cascade constructed from protocols described in the publication (see also Figure 2) to obtain validated fragments.

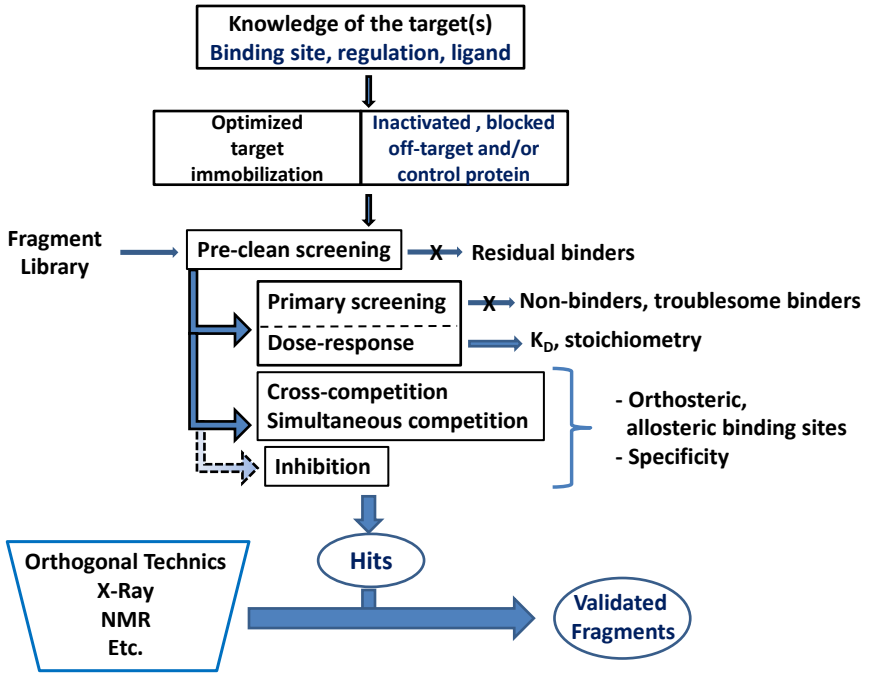


Table1. Main features of NMR, X-ray crystallography and SPR methods in Fragment Detection

Main biophysic technics	NMR		X-Ray crystallization		SPR
	ligand detection	protein detection	co-crystallization	soaking	
	STD and WaterLOGSY				
labeled	no	yes	no	no	no
heterogeneous[a]	no	no	no	no	Yes
direct -binding	yes	yes	yes	yes	yes
other binding assays	reporter-assay				competition; inhibition
multi-target binding assays (selectivity)	no	no	no	no	yes
protein MW	15-100 kDa	<30 kDa			<170 kDa[b]
KD determination	200µM to mM	µM to mM	no	no	>1000 µM
kinetic profils	no	no	no	no	yes
structural informations on binding site	yes for ligand	yes	yes[c]	yes	if competition
false+ and false- rates	high	low	low	low	low
time-consumming	medium	medium	high	high	low
protein-consuming	medium (0,5-5 µM)	high (10-500 µM)	high	medium	low[b]
DMSO concentration	up 10% d6-DMSO	up 10% d6-DMSO		up 10% DMSO[d]	3-5% DMSO
fragment-concentration	medium to high	high	high	high	low-medium
throughput	medium	low to medium	low	low	medium to high[e]
primary screening	yes	yes		yes	yes
information for optimization	from ligand	yes	yes	yes	yes

[a]immobilized -target

[b]favorized by low MW and high RI of fragment

[c] necessicity of 3D structure of protein

[d]higher than 10% DMSO could damaged the protein crystal and others possible solvents

[e] with recent technological improvement

Article highlights

- SPR devices are now state of the art for primary fragment screening
- Main advantages include low product consumption, rapidity and high sensitivity.
- SPR is a selective and informative method that allows detection and removal of false positive binders
- SPR is now validated for numerous challenging applications: GPCR, enzyme and PPI.
- The critical points are the immobilization of a functional target and a limited use of the selectivity