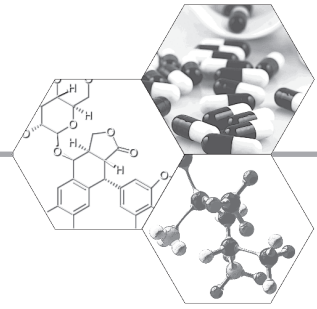

Emerging role of surface plasmon resonance in fragment-based drug discovery

Surface plasmon resonance (SPR) offers a method of biophysical fragment screening that is fast, efficient, cost effective and accurate. SPR is increasingly being adopted as a secondary assay to validate fragment hits. Recently, technical advances have resulted in the emergence of SPR as a primary screening methodology for fragment-based drug discovery. Moreover, SPR biosensor assays can be developed for a wide range of proteins, including membrane proteins, such as G-protein-coupled receptors. In this review, we discuss the advantages and limitations of SPR fragment screening including experimental consideration of reducing false positive and false negative rates to a minimum. We discuss how ligand efficiency can be used both as a method to eliminate false positives and to understand which fragments in a library may be a source of false negatives.

Fragment-based drug discovery (FBDD) [1], has emerged over the past 15 years as an important new approach to the discovery of lead molecules, several of which have now advanced far in clinical trials [2–4]. FBDD now ranks alongside structure-based drug design [5], analogue-based drug design [6] and high-throughput screening (HTS) [7,8] as one of the primary strategies for discovering novel drug candidates. The conventional approach to discover novel ligands for a drug target is by screening ten of thousands to millions of compounds in a high-throughput biochemical assay, with the aim of discovering ligands ('hits') with affinities in the nanomolar to micromolar range. The compounds screened in HTS can be selected from random, focused or diverse libraries of compounds of synthetic or natural products but are typically of molecular weights 350 to 500 Da (27 to 38 non-hydrogen or 'heavy' atoms). In contrast, the starting point for FBDD is the screening of a small library of low-molecular-weight compounds – 'fragments' – usually in the molecular weight range of 100 to 300 Da (8 to 23 non-hydrogen atoms) with affinities ranging from micromolar to millimolar [9]. Typically, most organizations undertaking fragment screening report using libraries containing only 500 to 2000 compounds, which, despite their small size, have proven amply sufficient to discover fragment hits for many classes of proteins, although some laboratories screen very large libraries of up to 24,000 by high-throughput methods [10]. One of the key tenets of fragment-based drug design is that a relatively small number of low-molecular-weight fragments can represent large areas of chemical space [11]. Low-molecular-weight compounds have been shown empirically to be less selective and capable of binding to a larger number of proteins than larger compounds [12]. As the molecular weight and, thus, complexity increases, the probability of an unfavorable interaction also increases [13]. The process of optimising an analogue and HTS-derived lead into drug candidates increases the molecular weight, on average by around 40 [14] to 80 Da [15], equivalent to an increase of 3 to 6 non-hydrogen atoms. The increase in the molecular weight and atom count during the optimization procedure is usually necessary to improve potency and selectivity of the initial hits. In contrast, an analysis of fragment-optimization projects for 30 protein targets revealed the ratio of the number of heavy atoms between the optimized leads and the fragment hit was 1.6, the golden ratio [16]. The golden ratio argument suggests that for less-druggable targets where the final compounds may posses a molecular weight of 500 Da and are composed of on average around 38 non-hydrogen atoms, the initial fragments hits could be up to 24 heavy atoms. With a larger number of heavy atoms required to optimize a fragment into a potent drug candidate, the optimization process could be considered daunting due to the increase in number of potential decision points in the design pathway. However, from its inception, FBDD has been intimately connected with structure-based drug design. The elucidation of x-ray crystal structures of the fragment–protein complexes have been crucial to the efficient prosecution of

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Key Terms

Fragment-based drug discovery: An approach to the discovery of low-molecular-weight small molecules that bind to a biological target. Fragment compounds are usually a fraction of the size of conventional leads and drugs. Due to their low-molecular-weight, fragment compounds often bind weakly, hence sensitive assay methods are required to detect their interactions with the biological target.

SPR biosensor: Optical biosensor instrument that exploits the phenomena of surface plasmon resonance in the detection of an interaction between biomolecules. The assays are configured with an analyte in solution and biomolecule immobilized on the surface plasmon resonance sensor surface.

many fragment optimization projects to produce lead compounds of nanomolar affinities from weak-binding fragments in only a few steps.

The low-molecular-weight of fragments result in low affinity interactions compared with conventional HTS-derived hits. Therefore, highly sensitive biophysical techniques, predominantly various NMR screening approaches [17–21] and direct observation by x-ray crystallography of crystals exposed to mixtures [22] and solutions [23] of fragments have been the screening methods of choice. High-concentration screening of very large fragment libraries using conventional biochemical assays have also been employed, utilizing existing HTS methods. However, the large false positive hit rate encountered in such assays usually required secondary biophysical screening to confirm and validate genuine hits. In recent years, the range of biophysical technologies applied to fragment screens has expanded beyond NMR and x-ray crystallography to include native mass spectrometry, isothermal titration calorimetry [24], protein thermal shift [25], affinity capillary electrophoresis [26], weak-affinity chromatography [27] and biosensor methods [28,29], such as surface plasmon resonance (SPR, Biacore), optical waveguide grating (Corning Epic) and reflectometric interference spectroscopy (ForteBio Octet). For the purposes of this review, we will focus on the most widely applied and what we consider to be the most promising optical biosensor method for fragment screening: SPR.

Advantages of SPR fragment screening

Surface plasmon resonance is a biophysical, label-free method for measuring molecular interactions. **SPR biosensors** measure the real-time interaction between molecules immobilized on the biosensor surface and molecules in solution. Immobilization of molecules to the biosensor surface can be undertaken using covalent (e.g., amine, thiol and aldehyde coupling) and non-covalent coupling methods (e.g., affinity tags, such as biotin streptavidin, monoclonal antibody tags and metal chelation). SPR is an optical biosensor detection method that measures the small changes in refractive index at the surface interface on binding event. SPR assays are capable of determining a number of parameters of the complex formation, including the association rate (k_a), the dissociation rate (k_d), the affinity (K_D) (by equilibrium or kinetic analysis) and the stoichiometry. By measuring the K_D over a temperature range it is also possible to determine the thermodynamic parameters of the interaction (ΔG, ΔH and ΔS) from the van't Hoff equation [30]. For further details on the physics of SPR and its experimental application to the measurement of biomolecular protein–ligand interactions, readers are directed to Huber and Mueller's excellent review on the subject [31].

The first applications of SPR biointeraction assays in FBDD emerged with the use of SPR as a secondary technique to confirm and validate fragment hits discovered by other screening methods [32–35]. The direct measurement of kinetics and affinity by SPR has a growing role as a confirmation method to complement other fragment-screening methods, but recently, SPR has been gaining a role as a primary fragment-screening technology [36–38]. A number of SPR fragment-screening campaigns, for a diverse range of proteins have been reported to date, including BACE-1 [33,39], MMP-12 [40], thrombin [41,42] and chymase [43], carbonic anhydrase II [42,44–46], HIV-1 protease [42], human serum albumin [42] and HIV-1 reverse transcriptase [47]. In addition, the first SPR fragment screens against GPCRs have also been reported for a stabilized adenosine A_{2A} receptor [48] and wild-type CCR5 [49].

Surface plasmon resonance-based biosensor screening is emerging a primary fragment-screening method [50] as it has several practical advantages over conventional NMR and x-ray crystallography-based fragment-screening methods.

First, SPR analysis provides a single method for both the HTS of fragment libraries and the confirmation and validation of the fragment hits. SPR biosensor interaction analysis is capable of both screening and validation of fragments as it provides a rich characterization of each fragment by providing accurate information on the affinity, kinetics and, if required, the thermodynamics of fragment–protein binding interaction. Kinetic analysis of fragment binding can indeed reveal fragments with slow dissociation rates [44]. Slow dissociation rates have been linked to superior clinical efficacy for many best-in-class drugs [51]. Therefore, fragments with slow dissociations rates may provide advantageous starting points for optimization. Thermodynamic data can also be derived from SPR analyses over a temperature range by employing the van't Hoff equation [30,52,53]. A large benchmark study found the equilibrium dissociation and thermodynamic constants

determined from the Biacore analysis matched the values determined using isothermal titration calorimetry [30], with the use of significantly less protein. Thermodynamic van't Hoff analysis of fragments by SPR may prove insightful given the arguments that fragments that bind with predominately enthalpic energies may make good starting points for optimization [54].

Second, SPR biophysical interaction assays can be developed far more quickly than other methods, particularly when compared with developing biochemical HTS assays or generating high-resolution, crystallographic systems. With the engineering of specific terminal tags on to the protein of interest, protein can be immobilized on the SPR chip surface from cell pellets or lysates and thus purified on the chip. If the protein is not tagged, direct coupling methods can be used to immobilize the protein on the biosensor surface, although the success of direct coupling assays is improved if the protein is purified to the high standards required for other biophysical techniques, such as crystallization. Alternative SPR assay formats to the direct-binding assays have been constructed for fragment screening to deal with the challenge of weak affinity and low molecular mass [55]. In a reverse arrangement, fragments have been screened against BACE-1 [33] using an assay configured using the method of probe competition-based inhibition-in-solution assays [55] where a probe compound that binds to a defined binding site on the protein of interest is immobilized on the sensor surface while the protein target is kept in solution at a constant concentration. Fragments are mixed with the target protein in solution in concentration-dependent manner and passed over the sensor surface. The concentration of free protein is measured by its interaction with the probe compound on the biosensor surface to determine fragment competition.

Third, SPR methods consume low amounts of protein. Typically, SPR fragment-screening campaigns, including assay development and hit validation, can consume as little as 25–50 µg of protein, which is at least ten- to 1000-fold less than other biochemical and biophysical fragment-screening methods, particularly when compared with protein-based NMR-by-structure–activity-relationship (SAR) methods and x-ray crystallography.

Fourth, the speed of SPR screening has increased. A new generation of SPR instruments, with greater throughput, enable biosensor-based screening for fragment libraries in a timeframe of around 2 days to 2 weeks for a typical size fragment library, depending on the number of concentration measurements screened, the stability of the protein, the size of the library and choice of instrument. In the best-case scenario, using stable, purified or tagged protein immobilized on a high-throughput Biacore 4000 instrument, with suitable standard ligands to aid assay validation, it is possible to discover and confirm fragment hits from a few micrograms of protein within a week.

Experimental limitations

To ensure fragment-screening experiments conducted using SPR biosensors provide the correct data it is important to understand the theoretical and experimental limitations of SPR screening [37]. The goal of proper assay design is to determine accurate binding data by reducing the false positive rate and the false negative rate as much as practically possible, to distinguish actual binding from non-specific binding, especially when running a high-throughput screen on large numbers of compounds [44,50]. An important factor in the success of an SPR fragment screen is the implementation of data quality control methods, the scaling and normalization of the primary-screening data to remove false positives and identify hits. Detail protocols on SPR fragment-screening methods have been described by Giannetti [50], and Navratilova and Hopkins [44].

The false-positive data detection rate can be reduced by referencing the data, to identify non-specific binding. An advantage that the majority of SPR technologies offer is the availability of multiple biosensor channels that can be used to screen multiple proteins in parallel. The parallel immobilization of the target, reference protein(s), including a blank channel as a reference surface enables the analyses' methods to distinguish between actual and non-specific binding [44]. The false negative rate of data detection is determined by a combination of the sensitivity of the biosensor instrument and sensitivity of the assay to detect a true binding event. The latest generation of SPR biosensors can detect small molecules with molecular weights as low as 50 Da. The detection limit is, however, influenced by the density of active protein attached to the surface. The activity is represented by R_{max} (maximum binding response) of ligand bound to the immobilized protein upon saturation of all binding sites on the surface while the affinity of the ligand binding is in correlation with affinity measured

by alternative assays. The magnitude of R_{eq} that can be recognized as the minimal detection level depends on the signal-to-noise ratio. Recently launched instruments, such as the GE Biacore T200 and GE Biacore 4000, can obtain quality responses at magnitudes below 1 RU (Response Units), due to very low noise levels. However, in fragment screening it is not only the noise of detection level that has to be considered, but also the noise of fragment-binding responses caused by binders and non-binders. In affinity-screening experiments, each fragment is injected at several concentrations ideally separated by blank injections of buffer, which are used to subtract injection drift in the system in order to obtain good quality responses. In fragment-screening experiments, blanks are usually injected every eight or more cycles of fragment injection, to increase the throughput of the screening. A lower number of blank injections can introduce issues with drift referencing or sample carryover and, therefore, increase noise of responses for binders and non-binders. In addition, many fragments have a high refractive index that can add to the noise level due to mismatch of refractive indexes between blanks and samples. Particular care needs to be taken with the sensitivity of SPR assays to DMSO. The high-refractive index of the DMSO solvent can be a major source of false positives if the concentration for samples and running buffer is not matched accurately [56]. Therefore, the minimal level of detection (RU response) in fragment screening has to be adjusted for each screen separately, the best based on binding of positive and negative controls during the screen.

An example of SPR fragment-screening data for SAP2k [44] is shown in **Figure 1**. A set of fragments was injected over target surface at three concentrations and positive and negative controls were injected during the experiment in order to check for stability of immobilized target and also at the beginning and end of the experiment at concentration series to demonstrate that the activity of target did not decrease over time. Increase of responses towards higher concentrations of fragments correspond to a higher false positive rate of fragments binding non-specifically to the target, which can be then subsequently eliminated by proper referencing.

The other aspect of sensitivity that has to be considered is binding the affinity of a fragment to the protein in combination with the molecular weight of the fragment. In order to saturate the surface with fragment, the fragment has to be ideally injected at significantly higher concentration than its affinity of binding. However, as many fragments have affinities at millimolar levels it is often not feasible to saturate the protein surface with fragment binding, as most of the fragments will aggregate and bind at concentrations exceeding 200 μM. Therefore, the signal responses are measured for concentrations at or far below their affinities. Based on the R_{max} of control compound binding and known screening concentration we can estimate what affinity of compounds can be detected at equilibrium R_{eq},

$$R_{eq} = \frac{R_{MAX} \times c}{K_D + c}$$

Equation 1

where *c* corresponds to a concentration at which fragments were screened.

For example, in **Equation 1** if R_{eq} = 5 RU is set as the cut-off limit for binders and non-binders, (as determined by the signal-to-noise ratio) and the screening concentration is 150 μM, and R_{max} at 25 RU, we can detect all compounds at affinities K_D = 600 μM and higher with confidence. The limits of detection can be increased by increasing R_{max} (e.g., by immobilizing a high density of receptor in its active conformation). Increased screening concentrations (c) may not necessarily improve sensitivity, as many fragments can bind non-specifically to the surface and, therefore, increasing the screening concentration may compromise specific binding. Therefore, both the affinity of ligand to the target protein and the molecular-weight detection limit of the biosensor instrument contribute to the sensitivity of SPR technology. The ability of SPR assays to detect binders with affinities far above the concentration being screened enables screens to be run at lower concentration than commonly used in many other fragment-screening methods. Screening fragments at low concentration (i.e., 16.6, 50 and 150 μM) eliminates much of the non-specific binding that is observed in screens run at higher concentrations. Non-specific binding can also be identified by screening against reference protein, in parallel. The shape of the sensogram itself also provides information on whether a binder is likely to be genuine. Finally, determining the behavior of a fragment in a dose-response experiment can identify compounds with super-stoichiometeric behavior, which can be a symptom of non-specific binding.

Initial hit selection, for single-concentration screens is typically decided by determining a sensitivity threshold several standard deviations above the normalized and scaled baseline response [50].

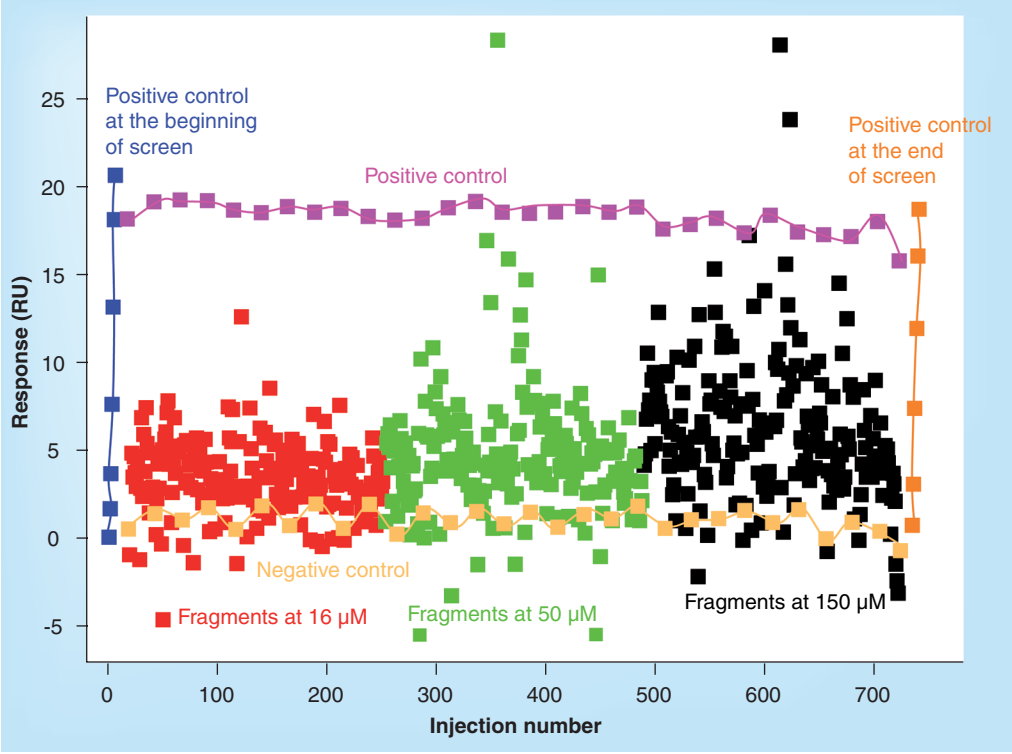

Figure 1. Example of SPR fragment-screening graph. Responses measured at equilibrium for fragments binding to the SAP2K protein are plotted versus injection. Each fragment is injected at three concentrations 16.6, 50 and 150 µM (red, green and black squares). Control compounds were injected during screen (positive (SB 220025), pink, and negative (furosemide), dark yellow) and a positive control was also injected at concentration series at the beginning of the screen (blue) and end of the screen (orange). Noise of responses is increasing toward higher fragment concentrations, however combination of data for each fragment at all three concentrations provides valuable information to distinguish between specific and non-specific binding (DATA NOT SHOWN). Experimental details of the fragment screen were previously published [44].

An alternative method to derive better sensitivity limits for detection that involve both the molecular weight and affinity of ligands, can incorporate the parameter of **ligand efficiency** (LE) [57] into the referencing method [44]. LE [57] is a metric commonly used in the fragment screening and serves as a guide for development of fragment hits into larger molecules. This term of normalized affinity, as expressed as the free energy of binding, by the number of non-hydrogen atoms was first described in literature by Kuntz *et al.* [58] and then implemented into a drug-discovery process by Hopkins *et al.* [57]. Navratilova and Hopkins have recently described a LE-based reference method for SPR fragment screening that combines both surface referencing from parallel channels and expected individual LE from each member of a fragment library [44]. The LE-based referencing method shows promise to eliminate the detection of false positives and thus increase the overall cost effectiveness of biosensor-based fragment screening [44].

The concept of LE can also be used to identify possible false negatives, that are most likely to fall below or above detection limits of SPR, as well as false positives. A comparison study of different NMR-based fragment-screening methods (saturation transfer difference and target-immobilized NMR screening) with SPR proposes a suitable SPR detection cut-off limit of K_D = 1 mM [59]. If our goal is to detect all fragments with LEs > 0.290, by combining LE and the detection limit of our SPR assay in terms of predicted affinities (e.g., 1 mM) we can determine which fragments are more likely to be missed (false negatives), at the set LE limit depending on the number of heavy atoms. Fragments with seven heavy atoms to achieve Fragments with seven heavy atoms need to bind with an affinity of at least K_D = 32 mM to achieve a LEs = 0.290 where fragments with 24 heavy atoms would have bind at, at least K_D = 7.8 µM affinity, at the same LE cut-off. If our cut-off detection limit is 1 mM measurable affinity

Key Term

Ligand efficiency: Binding efficiency per atom is a means of comparing the relative affinity of ligands of different sizes that bind to the same biomolecular target. Ligand efficiency (LE) is commonly defined as the ratio of Gibbs free energy of binding (ΔG) to the number of non-hydrogen atoms (N) of the compound:
LE = (ΔG)/N = -RTlnK$_i$/N.

calculated from **EQUATION 1**, at required LE 0.290 all fragments with heavy atoms 14 and higher would be detected **(FIGURE 2A)**, however we can miss fragments with lower heavy atom counts due to their weaker affinities necessary for binding at required LE **(FIGURE 2B)**. Therefore, this group can be larger source of false negatives and if we find fragments of this size that do bind, these would probably bind at higher LE than our cut-off limit. The minimal LE measured for fragments binding when the detection limit is set to 1 mM affinity can be then derived from **FIGURE 2C**. All parameters can be set accordingly to the criteria we require from a specific fragment screen. However, the benefit of SPR fragment screening is that even at a sensitivity cut of K_D = 1 mM, fragments below 13 heavy atoms that are detected will exhibit very good LEs, and will be detected when screened at concentrations a log order below the affinity cut-off limits. To decrease the number of fragments that could be possible source of false negatives, we would need to increase detection limits of the specific SPR assay or increase the LE cut-off. Another possibility lies in designing specific fragment libraries that can overcome false negative issues

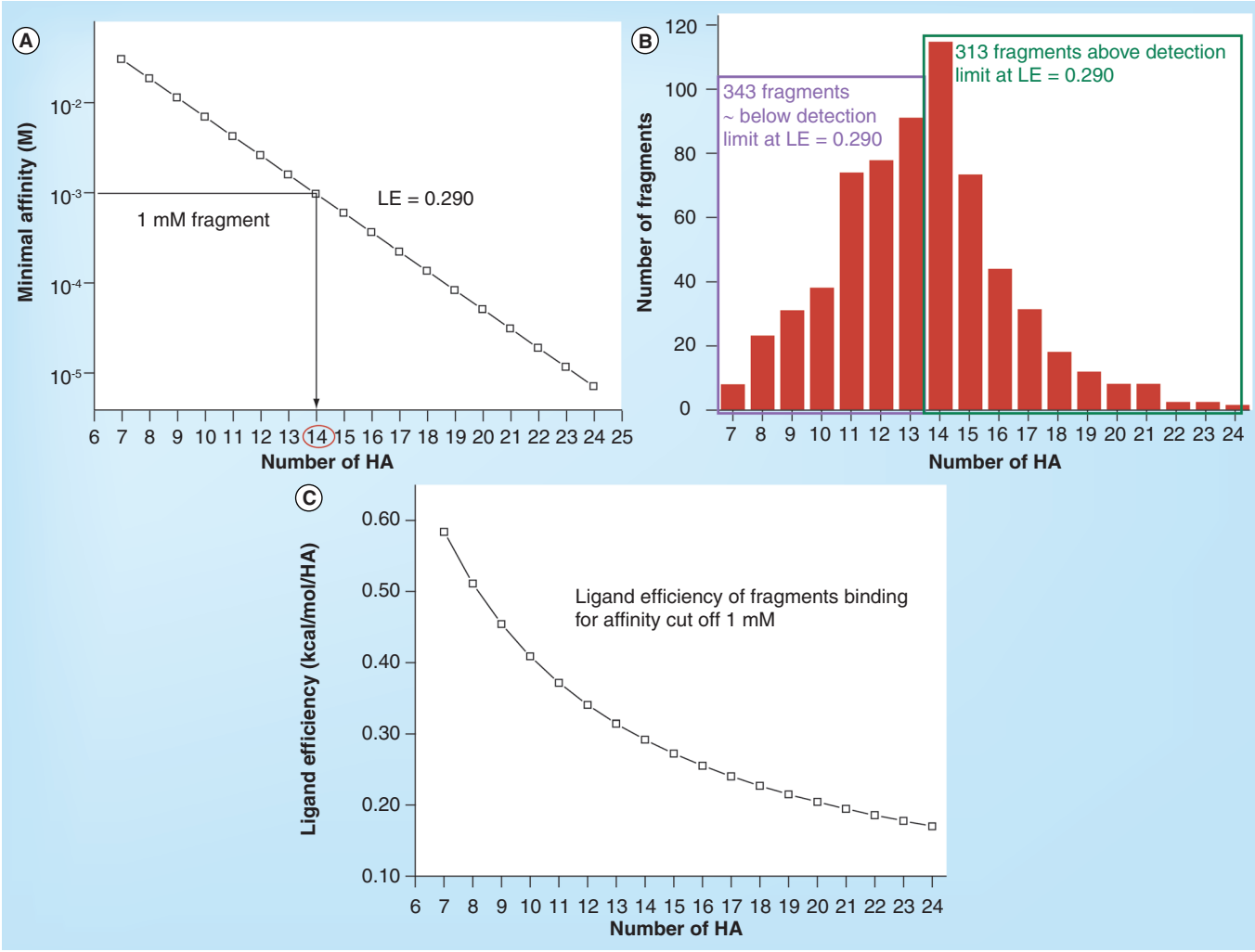

Figure 2. Ligand efficiency in fragment screening. (A) Graph showing relationship of binding affinity of fragments vs number of heavy (non-hydrogen) atoms for fragments binding at LE of 0.290 kcal/mol/HA at 25°C. Example of detection limit set to 1 mM resulting in the detection of fragments with 14 HA and more, with lowest ligand efficiency 0.290. **(B)** Example of fragment set of 656 fragments from library at University of Dundee and HA distribution (red bars). The set is divided into two groups, where 313 fragments would be detected at lowest ligand efficiency (0.290; green box; fragments with HA 14 and more) and 343 fragments could be missed at LE 0.290 (purple box; fragments with HA 13 and less), as these fragments can bind at weaker affinities in order to reach required LE, while these affinities are below the set SPR detection limits. **(C)** Representation of minimal LE measured for fragments if the detection limit of SPR is set to 1 mM affinity for each group of fragments based on the number of HA.
HA: Heavy atoms; LE: Ligand efficiency.

by identifying fragments that can be detected at minimal LE cut off. These libraries would contain a majority of fragments with larger heavy atom count preferably >13, depending on sensitivity limitations of SPR assays. Indeed, the argument for the application of LE to determine false negatives, proposed above, is not just applicable to SPR assay design but holds for any screening technology applied to fragment screening. Thus, depending on the fragment-screening technology employed, the optimal choice of fragment library may differ.

Emergence of GPCR fragment screening

The most exciting new area for the use of SPR-based FBDD is its emerging application to fragment screening for membrane protein drug targets, such as guanine nucleotide-binding protein-coupled receptors (GPCRs). Importantly, due to the need for soluble protein suitable for x-ray crystallography and NMR FBDD has not been routinely applied to membrane proteins. This is particularly limiting as 60% of all targets for approved drugs are located in the cell surface (compared with around 22% of all proteins in the human genome) with 27% of drugs targeting GPCRs [60].

Recent progress in the elucidation of x-ray protein crystal structures of several GPCRs [61] has created in the opportunity for structure-based drug design [62,63]. Moreover, the engineered stabilized receptors suitable for crystallization have also been shown to be suitable for SPR-based fragment screening. In a proof-of-concept study, a small focused library of approximately 50 xanthine derivatives and unrelated compounds (136 to 194 Da equivalent to 10 to 15 heavy atoms) were screened at a single concentration of 200 µM against an adenosine A_{2A} receptor [48] stabilized in an antagonist conformation [64]. The adenosine A_{2A} receptor was stabilized by the presence of four mutations (A54L, T88A, K122A and V239A) [64,65], engineered with a C-terminal His10 tag and immobilized on a Ni^{2+}–nitrilotriacetic acid chip. Eight hits were confirmed in concentration-series experiments to determine affinities ranging from K_D approximately 10 µM to 5mM [48].

However, improving the thermostability of GPCRs to achieve suitable protein for structural studies appears to be at the price of the wild-type pharmacology. The mutation process of achieving thermostability requires the receptor to be trapped in conformation induced by the test ligand, to produce either an agonist or inverse agonist/antagonist biased conformation [48,64]. Indeed, of the 27 mutations identified to stabilize the adenosine A_{2A} receptor with an agonist conformation, only three are in common with the 17 mutations required to stabilize the receptor in an antagonist conformation [64], indicating the mutations are specific for each thermostabilized conformation. The thermostabilized, biased conformational specificity has marked effects on the pharmacology of thermostabilized receptors compared with wild type [65]. Pharmacological binding studies of the antagonist thermostabilized adenosine A_{2A} receptor with standard pharmacological compounds using either radioligand competitor binding on transiently expressed receptor in HEK293T cells or SPR analysis of purified receptor, reveal antagonists displayed similar affinities to the wild type yet stabilized receptor was incapable of binding agonists with an appreciable affinity [65]. Antagonists binding to stabilized A_{2A} agonist binding mutants in contrast display a fivefold weaker affinity [64].

Thermostablized GPCRs can be purified and immobilized at densities high enough to enable SPR-based fragment screening, on Ni^{2+}–nitrilotriacetic acid chips, via the inclusion of His10 tag on the GPCR C-terminus [63,65]. The purified thermostabilized receptors are also amenable to crystallization studies, however GPCR fragment co-crystal structures are not yet routinely generated by high-throughput crystallography in a manner that has been routine for other proteins in FBDD projects [66,67]. Thermostabilized GPCRs are selected for bias conformations to detection of specific classes of ligands (i.e., stablized in agonist or antagonist conformations). In most lead-optimization projects the desired function is known and therefore a specific conformation could be stabilized. A potential limitation may be where alternative or novel modes of action are required for which there is no representative ligand to aid the stabilization selection process (i.e., orthosteric vs allosteric). Therefore, stabilized-receptor technology can be designed for the purpose of screening for specific function, however their application to the discovery of novel binding modes, such as novel allosteric sites, has yet to be reported. To maintain the full range of GPCR pharmacology and provide the opportunity for discovering multiple binding mechanisms and novel binding sites, it would be significantly advantageous to conduct biophysical fragment screening on receptors

with wild-type sequence and conformations. However, the disadvantage of using wild-type receptors is the challenge of optimising solubilization and assay conditions for intrinsically instable membrane proteins.

Recently, advances towards fragment screening of native GPCRs to discover allosteric site modulators have been reported with the SPR screening of CCR5 [49]. Navratilova *et al.* developed a high-throughput SPR assay for CCR5 on a Biacore 4000 instrument. The assay is based on immobilizing solubilized GPCRs. Solubilization conditions are selected by a systematic screen of detergent and lipids to identify buffer mixtures that maintain endogenous ligand binding on the immobilized receptor on the SPR chip [68]. The protein sequence of the target GPCR is wild type with the addition of the final nine amino acids from the rhodopsin C-terminal (C9) tag engineered on the C-terminus of the receptor. The GPCR is directly captured from the solubilized cell pellet material by the C9 interaction with the 1D4 antibody immobilized on the chip [68–71]. The C9–1D4 interaction is a highly specific and high-affinity interaction, thus the purification and immobilization is achieved in one step. To reduce the false-positive identification of non-specific binders, two reference surfaces were included in the assay: the 1D4 antibody alone and 1D4 with captured CCR5, blocked with the potent CCR5 antagonist, maraviroc **(Figure 3)**. In contrast to the thermostabilized GPCR SPR studies, the wild-type-CCR5 SPR assay has be shown to bind both small-molecule agonists [71] and antagonists [70,71]. For the screen, a library of 200 compounds was prioritized by Bayesian activity modeling from a larger library of over 90,000 compounds. The CCR5 Bayesian activity model was constructed using SAR data from the ChEMBL database [101]. The vast majority of the compounds in the selected set had molecular weights in the range 260 to 350 Da, equivalent to 20 to 27 non-hydrogen atoms, which, although larger than many commercial fragment libraries, is comparable to the 'rule of three' concept for fragment lead-library selection [72]. The screen identified five novel CCR5 ligands with affinities K_D = 8 uM to 49 uM. The binding of all five compounds was blocked by maraviroc. The LE for the hits are 0.24 to 0.335 kcal/mol/non-hydrogen atom, with an average LE = 0.282, which is comparable with that of maraviroc (LE = 0.272, K_D = 25 nM [71], 37 non-hydrogen atoms). The average number of non-hydrogen atoms for the five hit compounds is 23 (ranging from 20 to 24 non-hydrogen atoms). Interestingly, the ratio of non-hydrogen atoms for maraviroc (37 non-hydrogen atoms) to the average hit (23 non-hydrogen atoms) is 1.6, the golden ratio. In addition, the ratio of average non-hydrogen atoms of hits (23 atoms) to the additional structure required for a potent compound (14 atoms) is also 1.6. Therefore, the hit compounds discovered by the SPR screen can be considered as fragment ligands for CCR5 by the golden ratio argument for fragments leads [16].

To test the ability of the assay to discover compounds that bind to distinct allosteric sites, two CCR4 antagonists (pyrazinyl sulfonamides) with weak affinities for CCR5 were also included in the screen (19 and 20 non-hydrogen atoms). The screen measured the affinity of the pyrazinyl sulfonamides compounds as K_D approximately 10–29 μM with LEs of around 0.32 kcal/mol/non-hydrogen atom. Mutagenesis studies indicate the pyrazinyl sulfonamides bind to a distinct, intracellular allosteric site on the chemokine receptors [73]. Indeed the distinct mode of action of the pyrazinyl sulfonamides compounds is illustrated by the increase response when binding to maraviroc-bound CCR5 compared with apo CCR5.

The CCR5 screening results provide encouragement that GPCR fragment screening, even on wild-type receptors is technically feasible goal for SPR interaction assays. In order to achieve this goal a number of additional technical advances are required. The golden ratio size fragments for CCR5 are closer to the definition of 'lead-like' compounds [15,72] than what is usually considered 'fragment-like'. In order to detect typical fragment libraries comprising compounds ranging from 150 to 250 Da (11 to 18 non-hydrogen atoms) the sensitivity of the GPCR SPR assay may need to be improved to increase the R_{max} response for low-molecular-weight compounds. Increasing the density of active receptor on chip will increase the R_{max}. A number of parameters can be modified to increase the density of active receptor on the SPR chip, such as: (i) increasing the cellular expression level of the GPCR; (ii) systematically optimizing the lipid/detergent solubilization conditions, including exploring new detergents and additives or solublization methods such as liposomes; (iii) exploring alternative capture methods and chip types; (iv) and developing solvent-correction strategies to prevent the low-molecular-weight-fragments partitioning into lipid/detergent micelles. Solving these issues to further develop SPR GPCRs

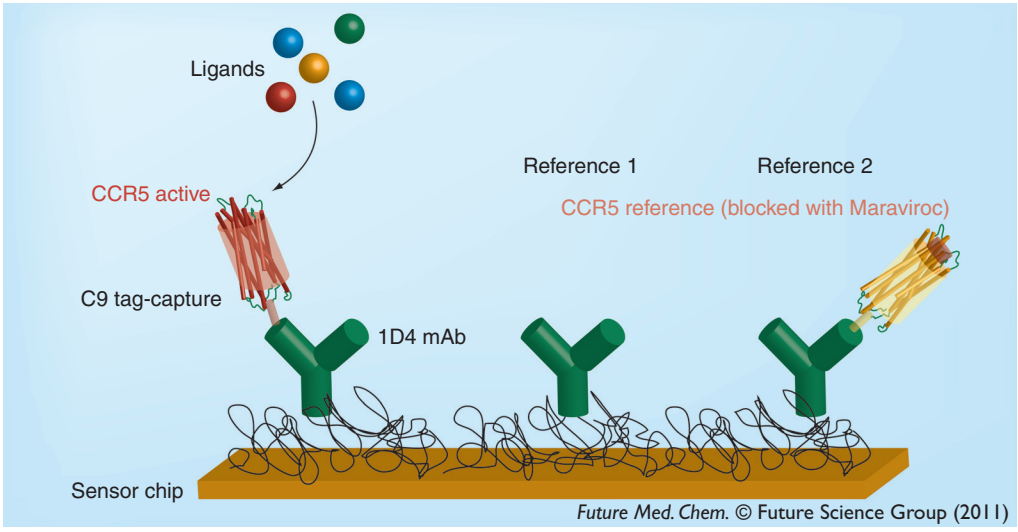

Figure 3. Example of experimental set-up for ligand screening on G-protein-coupled receptors. CCR5 was solubilized and captured via C-terminal C9 tag onto 1D4 mAb immobilized on the sensor surface. One-flow cell carried active CCR5, reference flow-cells contained 1D4 mAb only and also CCR5 with binding site blocked by Maraviroc. Ligands were then injected over all flow cells to determine binding.

assays offers the possibility of applying fragment screening to membrane receptors, to discover orthosteric ligands and even novel allosteric sites on GPCRs.

Future perspective

Surface plasmon resonance is emerging as an important technology in FBDD. The ability of SPR to accurately measure affinity, kinetics and, if required, thermodynamics of protein–fragment interactions has established SPR technology as an important secondary-assay technology to confirm hits. The advantages of SPR analysis in terms of speed of assay development and screening, low protein consumption and reliable data with low false-positive rates have resulted in SPR becoming a primary technology of choice for fragment screening. Indeed these advantages can significantly increase the cost effectiveness of FBDD in identifying ligand efficient hits, enabling a greater number of projects in a portfolio to benefit from fragment-based lead discovery.

Surface plasmon resonance offers a method of fragment screening that is fast, efficient, cost effective and accurate. Therefore, SPR is emerging a primary screening method of choice for fragment-screening projects. The speed and cost effectiveness of SPR fragment screening offers the opportunity for fragment screening to be undertaken very early in the drug-discovery process, as soon as tens of microgram amounts of purified or tagged protein are available. Analysis of successful fragment screens and optimization projects has shown the hit rate in fragment screens is indicative of the druggability [74,75] of a protein and the success rate of a large-scale HTS [76]. Thus, early fragment screening can be used as an index of target druggability to prioritize HTS hit-discovery project [76,77]. Given the cost effectiveness of SPR fragment screening and the large investment required for HTS assay development and screening, early-stage fragment screening of target proteins could increase the return on investment of a portfolio of HTS projects.

The emerging application of SPR fragment screening to membrane proteins, such as GPCRs, holds the greatest opportunity and challenge for the method. SPR fragment screening of thermostabilized GPCRs [48], where x-ray crystal structures are available, fits squarely within the current structure-based paradigm of fragment-based drug design [62,63]. However, the pharmacology of thermostabilized receptors can be biased [65]. In contrast wild-type receptors maintain their native pharmacology. The emergence of the screening of fragment-like compounds against solubilized, GPCRs immobilized via antibody capture appears to be applicable to the discovery of both orthosteric and allosteric sites on wild-type receptors [49]. The determination of experimental binding modes of fragments by structure-based methods is considered to be one of key requirements in fragment-based drug design [9].

However, structure-based drug-design methods can only be applied to stabilized GPCRs at present and not the instable, native receptors.

The emergence of SPR fragment screening against proteins for which crystal structures are not readily available creates both new challenges and opportunities for the development of the technology. The high quality of SAR data that can be generated for fragments with SPR assays opens up the possibility of using SPR-derived SAR data to build quantitative structure–activity models, that can be used for compound selection and design [78]. Therefore, the application of SPR to the generation of fragment SAR on structurally inaccessible proteins, in turn creates an opportunity to extend computational methods to fragment evolution based on ligand-based statistical design methods. Indeed, the use of SPR data to determine SAR for compounds from fragments through the optimization process is routine for soluble proteins for which x-ray structures are available [79]. As is often the case in science, the innovation in an experimental method in turn drives the need for innovation in the tools to exploit the data. SPR fragment screening provides drug designers with a rapid and cost-effective way to generate fragment SAR on a wide range of drug targets. The future of SPR fragment screening will, therefore, be linked to the development of new computational methods to exploit such data in drug design and, thus, create the next wave of innovation in fragment-based drug design.

Financial & competing interests disclosure

Navratilova and Hopkins are the founders of Kinetic Discovery Ltd, a contract research company specializing in biosensor-based screening. The authors have no other 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 apart from those disclosed.

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Executive summary

- Surface plasmon resonance (SPR) is a label-free biosensor-based method that can accurately analyze the association and dissociation rates of small-molecule interactions with biomolecules.
- SPR is emerging as a rapid and cost-effective method for screening small-molecule fragment libraries.
- Ligand efficiency-based referencing can be used to identify the sensitivity limits of false negatives and filter out false positives in SPR fragment screening.
- SPR methods are emerging that show promise in applying SPR fragment screening of membrane proteins, such as G-protein-coupled receptors.

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