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Affinity-based, biophysical methods to detect and analyze ligand binding to recombinant proteins: Matching high information content with high throughput

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ABSTRACT

Affinity-based technologies have become impactful tools to detect, monitor and characterize molecular interactions using recombinant target proteins. This can aid the understanding of biological function by revealing mechanistic details, and even more importantly, enables the identification of new improved ligands that can modulate the biological activity of those targets in a desired fashion. The selection of the appropriate technology is a key step in that process, as each one of the currently available technologies offers a characteristic type of biophysical information about the ligand-binding event. Alongside the indisputable advantages of each of those technologies they naturally display diverse restrictions that are quite frequently related to the target system to be studied but also to the affinity, solubility and molecular size of the ligands. This paper discusses some of the theoretical and experimental aspects of the most common affinity-based methods, what type of information can be gained from each one of those approaches, and what requirements as well as limitations are expected from working with recombinant proteins on those platforms and how those can be optimally addressed.

1. Introduction

Biophysical methods can have an impact in several valuable areas in early drug discovery. The available technologies have evolved in recent years such that the reliability, throughput, high-quality and orthogonality of approaches now compromise a tool-box of methods essential to modern drug discovery programs. Biophysics can allow rapid and reliable quality control checks on recombinant target proteins, and the assays in which they are used. This forms an important first step in establishing a suite of approaches focused on finding hits and leads.

While high-throughput screening of corporate compound collections has been the main approach used within the pharmaceutical industry to identify hits and leads, these methods have had limited success in identifying novel drug candidates. This fact, along with an increase in the number of biophysical approaches that can be applied, both to primary and secondary screening, as well as in lead optimization, has led the pharmaceutical industry to invest heavily in biophysical screening approaches in recent years. Some biophysical methods have the required throughput to compete directly with traditional biochemical screens such that they can be considered as truly primary hit finding assays. Yet more methods have sufficient throughput to allow focused screening for particular targets or for using selected compound libraries.

Although suitable for characterizing interactions of compounds covering a wide range of molecular weights, biophysical technologies are most often employed to focus on smaller libraries of low-molecular weight compounds. These so-called fragment-based lead generation approaches are being used increasingly, alongside or even instead of traditional high-throughput screening (see Albert et al. (2007) for a detailed review about the philosophy and strategy for fragment-based lead generation within AstraZeneca). The reasons for this are twofold: high throughput biochemical assays are already established as a route to screen larger compounds, but the probability of finding compounds showing optimal interactions is low; and perhaps more importantly, highly sensitive, high-quality biophysical assays are essential in order to detect the interactions of smaller compounds, due to their often weaker affinities. Given that the mean molecular weight of a drug molecule is around 335 Da, (median around 320 Da) and the mean molecular

Abbreviations: BACE, β-secretase; DBA, direct binding assay; DSC, differential scanning calorimetry; ED, equilibrium dialysis; EDC, N-ethyl-N′-(3-dimethylaminopropyl)carbodiimide; ESI, electrospray ionization; FAC, frontal affinity chromatography; HTS, high-throughput screening; IMS, ion mobility spectrometry; ISA, inhibition in solution assay; ITC, isothermal titration calorimetry; LC, liquid chromatography; MS, mass spectrometry; MW, molecular weight; NHS, N-hydroxysuccinimide; NMR, nuclear magnetic resonance; RU, resonance units; OWG, optical waveguide grating; SAR, structure activity relationship; SPR, surface plasmon resonance; TDC, target definition compound; TS, thermal shift.

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weight of a bioactive compound is 455 Da (median around 450 Da, see Tyrchan et al., 2009), biophysical methods capable of utilizing fragment start points with molecular weights in the range 150–250 Da may be an extremely useful primary screening approach. Identifying lower molecular weight start points may support several rounds of medicinal chemistry design-make-test cycles, during which the tendency is usually to increase molecular weight (Smith, 2009). This contrasts with using HTS approaches, which may successfully identify larger bioactive compounds, but the necessity will be to optimize compound properties with little or no change in molecular weight, in order to fit the profile for marketed drugs. Of course, this oversimplifies the situation somewhat, as there are many other considerations of compound properties that are important in drug discovery, although it does provide a convenient backdrop for the increased application of biophysical methods coupled to fragment screening.

Thus, biophysical methods are becoming increasingly established as complementary approaches to traditional hit finding routes, and are being actively exploited across the industry. The hope is that these biophysical methods will add an extra dimension to drug discovery by providing an opportunity to create hits and leads, rather than just finding them from within the corporate compound collection.

Thus, coupled to the incorporation of these new screening methods have been efforts to improve compound libraries for use with these technologies. These improvements include building fragment libraries which can be used as chemical start points, extended fragment libraries exploiting protein–ligand recognition principles, and target-specific focused libraries.

The result is that there are now a large number of method-library combinations which can be employed to monitor ligand binding in drug discovery.

In order to exploit these developments in technology and library design most effectively, it is necessary to consider the system and the information required before choosing which approach to use. Important considerations are the availability of the protein and well-characterized reference compounds, including the amenable concentration range, the functionality and the stability. The availability of tool compounds should also be investigated, as even if these are not essential, they may provide routes to additional valuable experiments for screening or evaluation. Different biophysical methods also offer a range of information content, so it is important to determine what information is critical to the stage of the project, and employ the most suitable method to extract that information from the collected data.

It may be necessary or desirable to combine approaches in order to identify and characterize compounds, to access the information required, in the most resource and time efficient manner. Consideration should be given to the most appropriate combinations of methods with the appropriate read-outs and level of confidence in order to achieve the desired goals. By combining techniques in this way, it should be feasible to provide medicinal chemists with data on the kinetics and thermodynamics of an interaction, which can then be interpreted alongside available structural information. This, almost full characterization of a binding event (lacking may still be the mean structures and in most cases the dynamic ensemble populations of one or both of the free interacting partners), should be invaluable in assigning some rules for guiding optimization of the appropriate parameters to meet the required candidate drug target profile.

So, the pharmaceutical industry is realizing that front-loading biophysical screening, or using it in conjunction with established HTS methods can be advantageous, as these methods can provide important information early in the drug discovery process about the required routes for lead generation for particular targets, and the potential success rates of HTS. This knowledge can be useful in helping to reduce the rate of attrition for valuable targets. It can also be useful in providing a more thorough description of protein–ligand interactions allowing attempts to optimize compounds towards profiles that appear to be favored in marketed drugs, for example larger negative enthalpies (Freire, 2008) and slower off-rates (Swinney, 2009).

This review highlights some of the available biophysical approaches that can be used to identify hits, provide data and information on the fundamental properties of the target protein–ligand interaction, and to give insights into how the thermodynamic and kinetic properties of that interaction may be modified in order to improve potency during the medicinal chemistry phase of a project.

2. Thermodynamic methods – ITC

Over the past decade ITC has been established as the gold standard method for directly measuring ligand binding affinity and thermodynamics (for a review see Freyer and Lewis, 2008). The technique often allows the affinity, enthalpy and stoichiometry of a binding interaction to be measured in a single experiment usually taking under one hour. Recent advances in sensitivity, reduction in cell volume, and automation have allowed the approach to evolve from a technique predominantly used for bespoke compound thermodynamic characterization, to one which can now begin to be applied in compound screening. The combination of thermodynamic and structural data has always been powerful in helping to guide molecular design, but the opportunity to characterize increased compound numbers relatively quickly, will see the use of ITC extended in medicinal chemistry design-make-test cycles.

The ITC experiment involves the monitoring of the heat produced (for an exothermic binding event) or absorbed (for an endothermic binding event) during the binding reaction (for a comprehensive protocol see Holdgate, 2010). As the name suggests, the experiment occurs at (almost) constant temperature with the ligand solution usually titrated from the injection syringe into the protein solution contained within the calorimeter cell. Modern calorimeters operate via power compensation, whereby the difference in the variable power, proportional to the binding heat, applied to the sample cell and the constant power applied to the thermal reference cell (in order to maintain a zero temperature difference between the two cells) is monitored by the instrument.

During the titration, in which small, typically 2–5 μL aliquots of the ligand solution are added, the first injections generate the largest heat change as the largest number of moles of protein–ligand complex are generated. As the titration progresses through subsequent injections, the protein becomes increasingly saturated with ligand, and the amount of newly generated complex falls (although the total amount of complex increases), resulting in a lower measurement of instrumental power. Finally, once all of the protein binding sites are occupied by ligand at the end of the titration, no further incremental complexation occurs and no further heat change is detected. Sometimes significant, non-zero heats following saturation are observed. These are often attributable to the heat associated with dilution of the ligand, as this is often larger than that associated with protein dilution (see Fig. 1).

Depending upon the binding affinity and the amounts of available reagents, it is often possible to arrange the experimental conditions so that a single experiment can provide precise estimates of the affinity (K_d), the enthalpy (ΔH) and the stoichiometry (n) of the binding interaction. This also allows calculation of the entropy (ΔS) from the Gibbs–Helmholtz equation:

$$\Delta G = \Delta H - T\Delta S = RT \ln K_d.$$