Sedimentation for success

Characterizing small-molecule/protein interactions by sedimentation analysis helps validate lead series early in the drug-discovery process.

BY MICHELLE ARKIN

The process of developing drugs is lengthy and expensive. Typically, the time from project initiation to FDA approval is 12–20 years at a cost of $400–800 million. Moreover, because drug development gets more expensive at later stages, critical decision points must be included early in the discovery process.

In most high-throughput drug discovery projects, drug leads are identified by the inhibition of protein function rather than the measurement of a binding interaction. While rapid, these assays will contain many “false positives,” including compounds that inhibit protein function because of protein precipitation or the formation of protein and/or compound aggregates. By contrast, compound series that demonstrate 1:1 binding to the protein—with binding affinity correlated to functional inhibition—are more likely to generate a successful lead series.

Sedimentation analysis, using tools such as the ProteomeLab XL-A/XL-I from Beckman Coulter, offers a straightforward approach for identifying artifacts and for characterizing binding stoichiometry and affinity early in the drug discovery process. This method measures the molecular weights (and therefore the association states) of molecules in free solution. In doing so, it can probe:

- protein heterogeneity,
- compound binding (dissociation and binding constants, as well as qualitative binding assessments),
- compounds and/or compound aggregation, and

These pieces of information can lead to the validation of a compound’s mechanism of inhibition and help prioritize compound series resulting from high-throughput screening.

Sedimentation toolbox

There are two general sedimentation analysis methods: sedimentation velocity (SV) and sedimentation equilibrium (SE) (1). SV is often used to characterize drug interactions with complex networks like tubulin. SE with UV–visible detection is the more commonly used method for measuring low-stoichiometry small-molecule/protein interactions.

SE measures the concentrations of components at equilibrium in solution. The readout from an SE experiment is an absorbance versus distance curve. The area under the curve is due to the absorbance of the sample, which is proportional to its concentration. The shape of this curve represents the balance of sedimentation and diffusion forces and is dependent on the molecular weight of the species undergoing sedimentation. SE is a quantitative method, but, qualitatively speaking, higher molecular weights lead to steeper sedimentation curves.

Figure 1 shows schematically how SE can be used to monitor proteins and compounds simultaneously. In this example, the protein is detected at 280 nm while the compound is analyzed at 300 nm. Note that the small molecule by itself shows no sedimentation curvature. The SE profile at 300 nm for the compound–protein mixture, by contrast, shows curvature similar to that of the protein; thus, the apparent molecular mass of the bound small mole-
One SE experiment requires approximately 100 µL of sample, and a typical small-molecule/protein analysis uses 1–5 samples. Thus, the amount of material required for characterization is comparable to (or less than) what is usually used for biophysical methods such as calorimetry or nuclear magnetic resonance (NMR) spectroscopy. The experiment is nondestructive, and samples can usually be isolated after the SE experiment is complete. Finally, unlike many methods, the protein and small molecule are detected at the same time; thus, both binding and lack-of-binding are observed in this suite of experiments.

**Analysis in action**

SE can impact early-stage drug discovery activities in several ways. One function is simply to demonstrate whether a compound binds to a protein. For example, Sunesis Pharmaceuticals identified a small-molecule inhibitor of the tumor necrosis factor (TNF) α/TNF receptor complex by screening, but it was not clear from the inhibition data whether the compound bound to the hormone or the receptor (2, 3). Addition of the compound to equimolar amounts of each protein (Figure 2) clearly demonstrated that the compound bound selectively to the TNFα receptor.

SE has also been used to assess the oligomerization state of a protein in the presence of small-molecule inhibitors. In some cases, compounds act as oligomerization inhibitors, shown by SE as a quantifiable reduction in the curvature of the protein profile. In other cases, compounds cause inhibition by aggregating the protein. This can be observed as an increase in the curvature of the protein profile upon addition of compound. Finally, any curvature of the compound profile in the absence of protein (or in the presence of a small amount of protein) is likely due to aggregation/self-association of the compound.

The combination of approaches efficiently distinguished between the series that was more likely to yield a druglike inhibitor—rapid binding and dissociation kinetics, stoichiometric properties of the two series. Surface plasmon resonance (SPR) measurements gave information about the kinetics and stoichiometry of compound binding to immobilized protein, and SE identified the protein aggregation state of PTP1B in the solution phase. The data indicated that series A acted by slowly causing aggregation of the protein—explaining the sensitivities to both time and protein concentration. By contrast, series B demonstrated properties consistent with a druglike inhibitor—rapid binding and dissociation kinetics, stoichiometric binding to protein, and the absence in a change of the protein’s aggregation state. The combination of approaches efficiently pointed the drug discovery effort toward the series that was more likely to yield results.

**Detailed binding insight**

For compounds that bind tightly to protein, SE can assess the stoichiometry of binding. If the dissociation constant (K_d) for the interaction is lower than the concentration of protein and compound (i.e., less than ~1 µM), essentially all of the compound will bind to protein until the binding sites are saturated. Throughout this part of the titration, the curve shown at the right in Figure 1 will get steeper, without a significant increase in the Y-intercept. After binding saturation, all excess compound will remain unbound. This will be observed in SE analysis as an increase in the Y-intercept of the sedimentation curve without a change in curve shape. This experiment does not differentiate between “irreversible” and rapidly reversible binding interactions.

Going a step further, SE can be used to obtain a good estimate of binding affinity for K_d values in the micromolar range. Several approaches have been described, but most of the literature focuses on the association of two biomolecules, such as protein–protein or protein–DNA interactions. One of the approaches that has been demonstrated for protein/small-molecule interactions uses software called Hetfitter (5). For this method, SE data are collected for

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**Binding affinities for small-molecule inhibitors of interleukin-2**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Inhibition assay</th>
<th>SE binding assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound 1</td>
<td>280 µM</td>
<td>400 µM</td>
</tr>
<tr>
<td>Compound 2</td>
<td>26 µM</td>
<td>50 µM</td>
</tr>
<tr>
<td>Compound 3</td>
<td>3.5 µM</td>
<td>7.2 µM</td>
</tr>
</tbody>
</table>

*a* Binding determined by sedimentation equilibrium (SE) agrees with functional inhibition (5, 6).
three compound-to-protein concentration ratios at, at least, one wavelength where the small molecule absorbs light. Additional wavelengths and multiple sedimentation speeds can help validate the results. The software determines the best-fit $K_d$ using user-input reagent concentrations and molecular weights. Depending on the sample preparation, 2–6 $K_d$ measurements can be obtained per 24-hour experiment. For a series of small-molecule ligands binding to interleukin-2, the $K_d$ values predicted by Hettfitter were well-correlated with the inhibition constants obtained from a functional assay (see the table; 5, 6).

**Focusing efforts**

In the practice of early-stage drug discovery, a number of methods are brought together to validate compounds for further optimization. The degree of detail necessary to make an effective decision depends upon many factors, including the novelty of the compound and biological target, the accuracy of the discovery assay, and the perceived difficulty of finding inhibitors of the desired target. SE can play an important role in helping to set priorities and to identify those compounds most likely to succeed.

At Sunesis, we applied SE in a quantitative mode to characterize inhibitors of the IL-2/IL-2 receptor interaction. Protein–protein interactions such as IL-2/IL-2 receptor have been very difficult to inhibit with small molecules, and compounds identified through functional inhibition are sometimes found to be artifacts. Therefore, inhibitors of IL-2 were tested by SE, NM, SPR, and X-ray crystallography to determine whether they displayed 1:1 binding stoichiometry and binding affinities similar to functional inhibition constants. In these two drug discovery programs, as in others, sedimentation analysis has improved the efficiency of drug discovery by helping to focus efforts on those compound series most likely to succeed.

**References**


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