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NLM Citation: Auld DS, Inglese J, Dahlin JL. Assay Interference by Aggregation. 2017 Jul 26. In: Markossian S, Grossman A, Arkin M, et al., editors. Assay Guidance Manual [Internet]. Bethesda (MD): Eli Lilly & Company and the National Center for Advancing Translational Sciences; 2004-.
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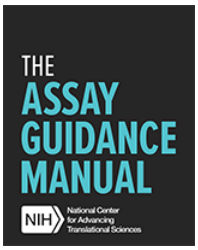

Assay Interference by Aggregation

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Created: July 26, 2017.

Abstract

Aggregation is a common mechanism of compound-mediated assay interference encountered in high-throughput screening (HTS) and follow-up experiments. Compounds that form aggregates *in situ* can nonspecifically perturb biomolecules in biochemical and cell-based assays. Nonspecific bioactivity from aggregation can waste significant resources when unaccounted for in assay design and readout interpretation. This chapter describes two general principles: (a) experimental considerations to mitigate the impact of aggregation in bioassays, and (b) counter-screens and other strategies to identify aggregation among bioactive test compounds. This content should be useful for those performing bioassays utilizing small-molecules, including HTS and follow-up assays, chemical biology, and molecular pharmacology.

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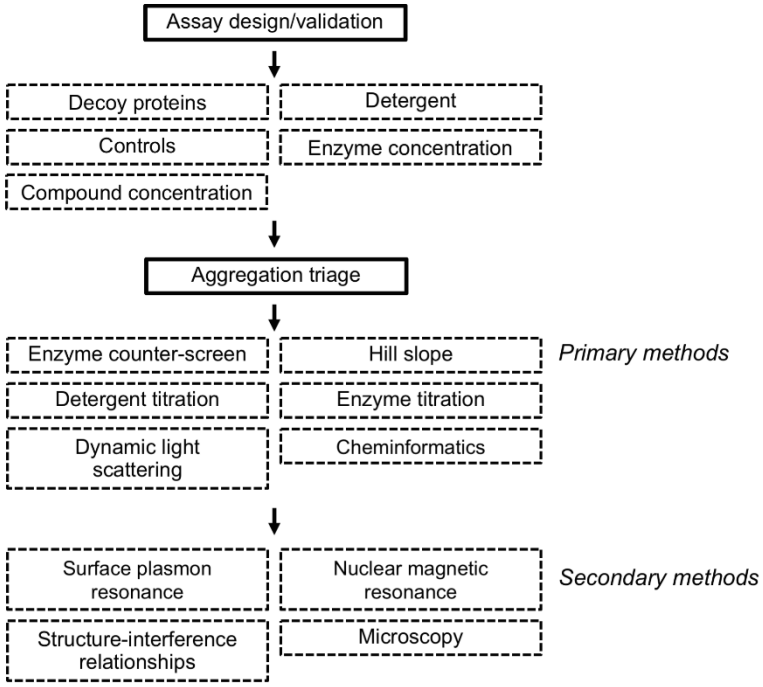
Flowchart



Abbreviations

BSA	bovine serum albumin
CAC	critical aggregation concentration
CRC	concentration response curve
CMC	critical micelle concentration
DLS	dynamic light scattering
HAT	histone acetyltransferase
HTS	high-throughput screening
IC_{50}	half-maximal inhibitory concentration
K_d	dissociation constant
MLSMR	Molecular Libraries Small Molecule Repository
NMR	nuclear magnetic resonance
qHTS	quantitative HTS
SIR	structure-interference relationships
SPR	surface plasmon resonance
TEM	transmission electron microscopy.

Introduction and Background

Introduction

Drug and chemical probe discovery often utilizes real and virtual high-throughput screening (HTS) to identify chemical matter for subsequent optimization. HTS readouts from biochemical and cell-based systems are subject

to a variety of compound-mediated assay interferences, including aggregation (1). The apparent bioactivity derived from aggregation is often difficult to optimize, and if not recognized early in the discovery process, it can result in significant wasted resources and questionable conclusions derived from these experiments. This section provides an overview of the prevalence and mechanistic details of aggregation in HTS.

Mechanism of Assay Interference by Aggregation

Understanding the mechanisms of aggregation is important because it can inform data interpretation and counter-screen design. Aggregation occurs when susceptible test compounds form aggregates in solution (colloids) (**Figure 1**). For these compounds, aggregation occurs at a critical aggregation concentration (CAC), which is typically in the low-to-mid micromolar compound concentration range. Unlike simple compound precipitation, aggregates can dissolve when diluted below the CAC (2). These solid colloids are composed of up to 10^8 small-molecules and are several hundred nanometers in mean diameter (n.b. some polydispersity), present in mid-femtomolar concentrations when formed, and are therefore not typically observed by visual inspection like compound precipitation (2,3). For enzyme systems (the most well-characterized system for aggregators), bioactivity results from a *reversible* association between enzyme and aggregate surface (4,5). It is currently thought that this binding represents protein *ad*sorption, rather than *ab*sorption, with activity modulation occurring by partial protein unfolding (6). This nonspecific binding typically results in enzymatic inhibition. Furthermore, the affinity of aggregate-protein binding is quite strong, with K_d values in the picomolar range. By contrast, aggregates do not appear to have high affinities for other biomolecules such as DNA or peptides (3). However, aggregates may still interfere with protein-DNA/RNA binding assays through sequestration of the protein component. Individual aggregates can bind up to 10,000 individual enzymes (2). Interestingly, proteins bound to aggregates can retain their activity upon dissociation ("catch and release") (5).

Notably, certain compounds may form "non-classical", *smaller* aggregates capable of modulating protein structure and function. For example, the small-molecule JNJ525 showed apparent low micromolar activity in a TNFα-TNFR1/2 TR-FRET protein-protein interaction assay (7). Like conventional aggregators, this compound showed detergent-sensitive activity and steep Hill slopes in the primary biochemical assay. Detailed mechanistic studies including x-ray crystallography revealed JNJ525 binds TNFα as an ordered 5-member aggregate, disrupting the TNFα-TNFα protein-protein interaction and preventing TNFα-TNFR1/2 association.

Importantly, aggregation can be dependent on multiple experimental factors, including the structure of the test compound, the assay conditions, and the susceptibility of a given target system to aggregates. For example, it is well known that the CAC for aggregators is compound-specific (e.g., some compounds may aggregate at 5 µM, whereas others may only aggregate at 50 µM compound concentrations). In a series of surface plasmon resonance (SPR) experiments, aggregates showed compound-dependent behavior versus the same target (8). In other words, not all aggregates behave the same way versus the same target. Furthermore, studies of the same aggregators versus different targets demonstrate proteins have different susceptibilities to aggregation (9,10).

Such varying responses to different aggregates may reflect differences in specific compound-aggregate affinities. For instance, a given aggregate may have protein-dependent affinities, as recent reports with dye-stabilized compound aggregates demonstrated up to 90-fold differences in apparent affinities between four unrelated proteins (3). These varying responses may also be explained by the nature of specific protein dynamic perturbations induced by a given aggregate. Depending on the tertiary structure of a given protein, aggregate adsorption may significantly perturb protein structural dynamics and function, while in other cases it may cause no appreciable changes.

Prevalence of Aggregation in HTS

Aggregation is a significant source of nonspecific bioactivity, particularly in HTS. In a seminal study screening a 70,563-member Molecular Libraries Small Molecule Repository (MLSMR) library for AmpC β-lactamase

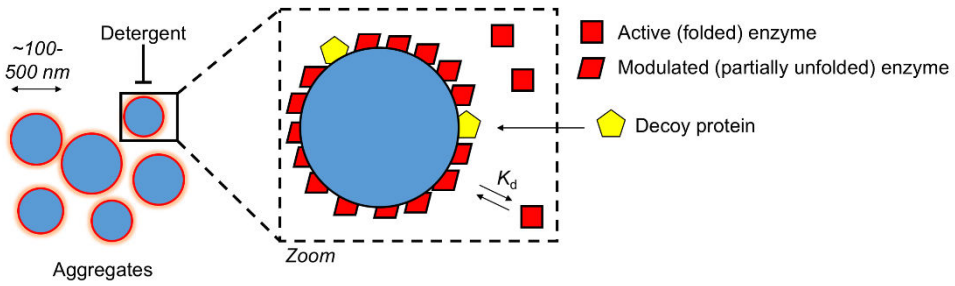


Figure 1. Current model of aggregation. Above the CAC, compounds form solid aggregates, typically 100-500 nm in mean diameter. Enzyme then adsorbs to the aggregate surface with high affinities (e.g., K_d = picomolar to nanomolar ranges). Adsorbed proteins are partially unfolded, which can modulate activity. Free, unperturbed protein occurs when the aggregates become saturated. When present in sufficient concentrations, detergents can disrupt aggregates. Decoy proteins such as BSA can compete with enzyme for adsorption to aggregate surfaces

inhibition, 1204/1274 (95%) of the *primary actives* were identified as aggregators (10). Overall, 1.7% of the entire library was flagged as a likely aggregator in this HTS due to detergent-sensitive bioactivity. In another MLSMR HTS for cruzain inhibition, 1.9% of the 197,861-compound library and approximately 90% of the primary actives showed detergent-sensitive inhibition, behavior consistent with aggregation (11). Based on these studies, one could estimate that compounds capable of forming aggregates constitute less than 5% of HTS libraries. These studies also demonstrate that if an assay is not properly designed, the vast majority of primary actives can result from aggregation. The main consequences of enriching active compounds with aggregation is that significant resources are spent chasing aggregates in place of potentially more tractable chemical matter.

Section Summary

Aggregation represents a significant source of nonspecific bioactivity and compound-mediated assay interference in HTS and related experiments. Aggregation occurs at compound- and assay-specific critical compound concentrations, and promotes nonspecific activity through partial protein unfolding. Understanding the mechanism of aggregation allows for (a) strategies to mitigate the incidence of aggregation interference, and (b) the design of counter-screens to identify bioactivity due to aggregates.

Strategies to Mitigate Incidence of Aggregation in Biochemical Assays

Introduction

This section describes several experimental strategies and technical considerations to reduce the incidence of aggregation in biochemical assays (n.b., strategies for cell-based assays are less characterized). Strategies discussed include the use of detergents and decoy proteins to prevent aggregate formation, the choice of enzyme and compound concentrations to mitigate the effects of aggregates on assay readouts, and control compounds to characterize the effects of aggregation on assay readouts.

Detergents

Perhaps the single-most effective strategy to prevent aggregation is to include detergents in assay buffers (4,9,12,13). These reagents act by disrupting colloid structure and can raise the CAC. With most aggregators, activity is dramatically attenuated by the inclusion of detergents (**Figure 2**). In many instances, the addition of detergent can also reverse nonspecific protein modulation by aggregation (4). Another benefit of including detergents is the prevention of nonspecific protein adsorption to container walls, thus preventing time-dependent losses in enzyme activity and decreasing the amount of required enzyme.

There are several choices of detergents to prevent colloid formation, with most being nonionic (**Table 1**). The most popular and well-characterized detergent with respect to aggregation is Triton X-100, which is often used at 0.01% (v/v) final concentrations. A common misconception is that using these "default" detergent conditions will completely eliminate assay interference and nonspecific bioactivity from aggregation. While this concentration is a helpful starting point, it does *not* absolutely prevent colloid formation for every compound. Detergents vary greatly in properties (**Table 1**) and detergents such as Triton X-100 can form very large micelles at concentrations at or above the critical micelle concentration (CMC) which are difficult to remove from proteins. Therefore, detergent concentrations that are well above the CMC should be avoided and one should verify that the complete assay system (e.g., target, reagents, readout, instrumentation) is compatible with the proposed detergent under the conditions to be tested.

A representative example of detergent effects on the distribution of HTS actives can be seen in a comprehensive study exploring the mechanistic basis of the actives from a β-lactamase HTS (15). Detergent sensitivity was used to establish the preponderance of actives that were a result of aggregation, with remaining mechanisms consisting of nonspecific reactive compounds, PAINS-like compounds, and *bona fide* AmpC β-lactamase inhibitors, the latter comprising < 2% of the HTS actives (**Figure 3**) (1,15,16).

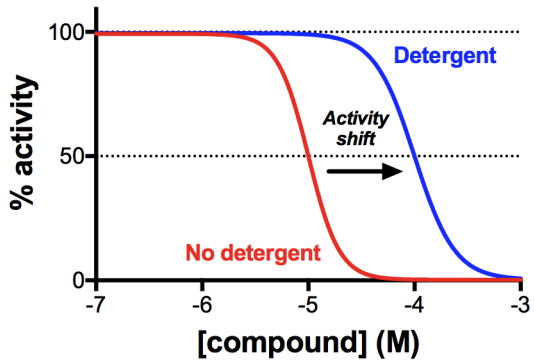


Figure 2. Detergent-dependent activity of aggregators. Shown is a simulation of a prototypical aggregator assayed without (red line; IC_{50} = 10 μM) and with detergent (blue line; IC_{50} = 100 μM). Sufficient amounts of detergent will typically attenuate bioactivity due to aggregation, as illustrated by the activity shift.

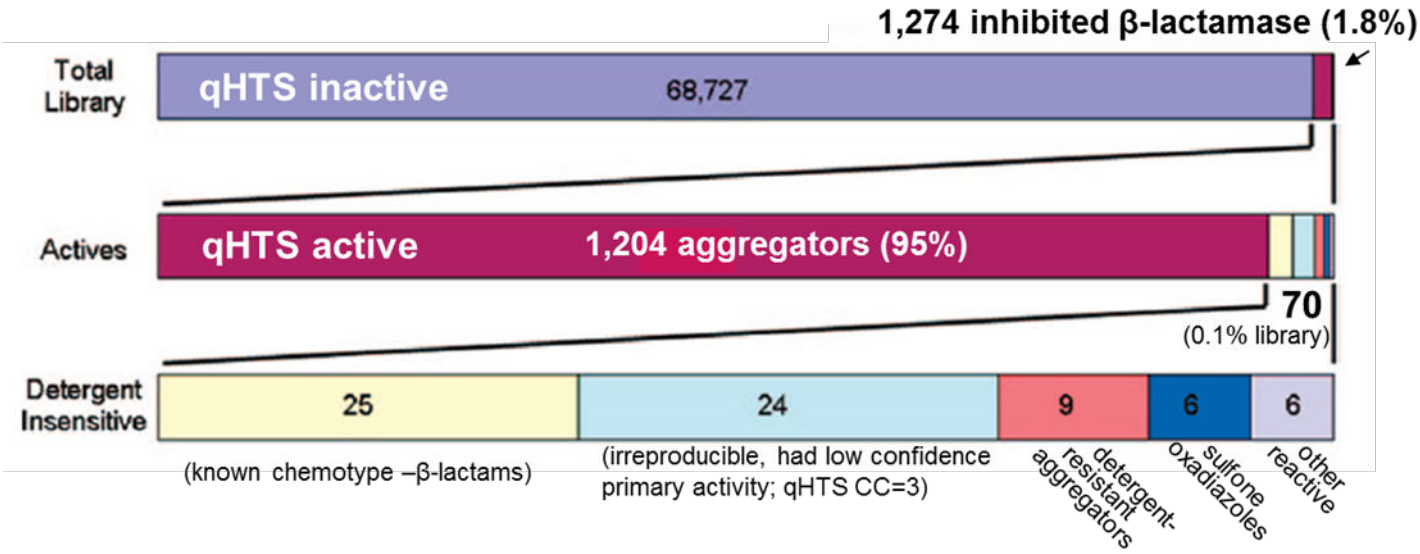
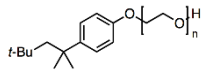
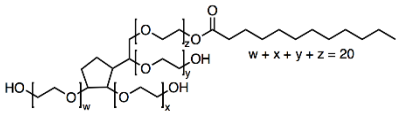
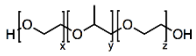
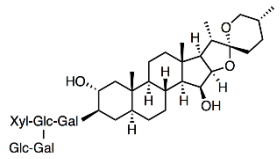
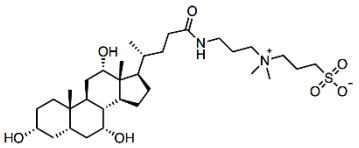


Figure 3. Mechanistic analysis of actives from a β-lactamase biochemical HTS dominated by aggregators. Using quantitative HTS (qHTS), ~70 K compounds were assayed for β-lactamase inhibition. The active compounds (1.8%) were analyzed for detergent-sensitive inhibition, which accounted for 95% of the primary qHTS actives. The remaining 5% of detergent-insensitive compounds (70 compounds) included β-lactam-based inhibitors, irreproducible samples, nonspecific reactive substances, and detergent-resistant aggregators. Detergent-resistant aggregators comprise only 0.7% of the aggregators identified. (adapted with permission from K Babaoglu *et al.* J Med Chem, 2008) (15)

Table 1. Example detergents used in biochemical assays. CMC, the critical micelle concentration, or the concentration at which the detergent begins to form micelles. Aggregation number, the number of detergent molecules in a micelle.

Detergent	Chemical structure	Ionic / nonionic	CMC[a]	Detergent aggregation number	Technical notes
Triton X-100		Nonionic	0.2-0.9 mM (20-25 °C) 0.01% = 0.16 mM	100-155	Often used at 0.01% (v/v) or higher; can produce H_2O_2 in aqueous solutions (14) (recommend preparing fresh buffers for experiments)
TWEEN-20		Nonionic	0.06 mM (20-25 °C) 0.001% = 0.8 mM		
Pleuronic F-68		Nonionic	0.04 mM (20-25 °C) 0.01% = 0.1 mM		
Saponin	Variable	Nonionic			
Digitonin		Nonionic	< 0.5 mM (20-25 °C)	60	
CHAPS		Ionic	6 mM 0.01% = 1.6 mM	10	

[a] Data obtained from manufacturer (Sigma-Aldrich)

Decoy Proteins

Another tactic to mitigate the effects of aggregation is the addition of decoy proteins. The current model for this phenomena involves pre-saturation of aggregates by relatively high concentrations of decoy protein, leaving the biomolecule of interest (e.g., target enzyme) unperturbed by the aggregates.

The prototypic example is the carrier protein bovine serum albumin (BSA). High concentrations of BSA can prevent nonspecific enzymatic modulation by several known aggregators when BSA is present in the assay before the addition of aggregators (17). Importantly, high concentrations of BSA do not routinely reverse enzyme modulation (17).

Several important technical points should be noted:

- A suggested starting concentration for BSA is 0.1 mg/mL.
- Because they do not reverse bioactivity from aggregation, BSA or other proteins should be present in the assay system *before* the addition of test compound.
- Carrier proteins have the potential to sequester monomeric test compounds, lowering the free test compound concentration. This effect may be pronounced at high protein:compound ratios.
- Verify high concentrations of aggregate decoy proteins such as BSA do not interfere with the assay readout or disrupt assay performance. For example, free thiols on untreated decoy proteins may react with assay reagents or electrophilic test compounds.

Enzyme Concentration

For enzymatic biochemical assays, another proposed tactic to mitigate the effects of nonstoichiometric bioactive compounds (such as aggregators) is to simply increase the concentration of target enzyme (18). This approach is based on a proposed stoichiometric model of enzyme inhibition for aggregators (**Equation 1**) (19):

$$\frac{[\mathrm{I}]}{K_\mathrm{d}} = \frac{\text{inh }\%}{100 - \text{ inh }\%} + \frac{\left[\frac{(\text{inh }\%)}{100}\right][\mathrm{E}]}{K_\mathrm{d}} \qquad \text{Equation 1}$$

In this model, enzyme inhibition will appear stoichiometric in cases when enzyme concentration significantly exceeds the K_d value of the inhibitor. This is not an unreasonable assumption for aggregators (recall that many aggregators appear to have K_d values in the low picomolar range). In other words, the IC_{50} value of an aggregator will increase linearly with increases in enzyme concentration. In this model, non-aggregating compounds are not expected to have such dramatic shifts in IC_{50} values unless they have low K_d values, which is unlikely when screening non-optimized chemical matter versus a novel target.

This technique is not without its drawbacks. Aside from consuming more enzyme per test compound, a potential downside to this tactic is that by increasing the amount of enzyme, reactions will proceed faster (18). A practical consequence is that it may be more difficult to sample reactions at low substrate turnover/product formation.

Compound Concentration

Another approach to prevent the formation of aggregates is to decrease the concentration of test compound. The logic of this approach is to lower compound concentrations below the CAC, so that observed bioactivity would more likely be due to other mechanisms of target engagement. This approach will only prevent colloid formation if the compound test concentrations are below the CAC for the specific experimental condition tested, and may not be effective for compounds with relatively lower CACs. Another drawback to this approach is the increased probability of missing less potent but potentially useful compounds which may only be identified at higher compound concentrations. This dilemma can be addressed by using qHTS should a range of compound concentrations be necessary to limit the false-negative occurrence or comprehensively profile the chemical library (20).

Controls/Reference Compounds

During assay development and optimization, it may also be advantageous to assess the effect of known aggregators on assay readout (**Table 2**) (21).

This tactic can help guide the optimization of experimental conditions to reduce the incidence of aggregation. For example, if aggregator controls show significant bioactivity, one may wish to modify the concentration(s) of detergent, aggregator decoy proteins, test compounds, and/or enzyme.

A second potential benefit of this approach is that it permits the actual characterization of aggregation on the assay readout. In conventional cell-free enzymatic assays, this effect may be as simple as enzyme inhibition marked by steep concentration-response curves (CRCs). This approach may be especially useful in more complex assays such as multi-step biochemical assays and cell-based assays, where the potential effect(s) of aggregation may not be intuitive. For example, some aggregators exhibit "bell-shaped" CRCs in cell viability assays, a phenomenon which reflects the concentration of bioactive, monomeric compound (**Figure 4**) (22). Taking the time to robustly characterize the effect of aggregation on assay readout may enhance bioactive compound triage. That is, when test compounds produce a similar readout to known aggregators, one may wish to triage such compounds or flag them for subsequent counter-screens (see section "STRATEGIES TO IDENTIFY AGGREGATION").

A third potential benefit of using aggregator controls is that it may help estimate the incidence of aggregation among bioactive compounds. If a variety of known aggregators are inactive, one might anticipate aggregators would not be particularly enriched among the primary active compounds. On the other hand, if most aggregator controls are active, one might anticipate a formidable proportion of the primary active compounds may owe their bioactivity to aggregation. This estimate may inform the design of the post-HTS screening tree (23). In the latter scenario, one may wish to perform a series of aggregation counter-screens earlier in the triage process, in order to rapidly discard nonspecific aggregators.

Aggregation can be dependent on the assay conditions, meaning even known aggregators will not form micelles in every assay. Therefore, if using this approach, it is advised to include several different controls to get a more comprehensive assessment of aggregation in a particular assay.

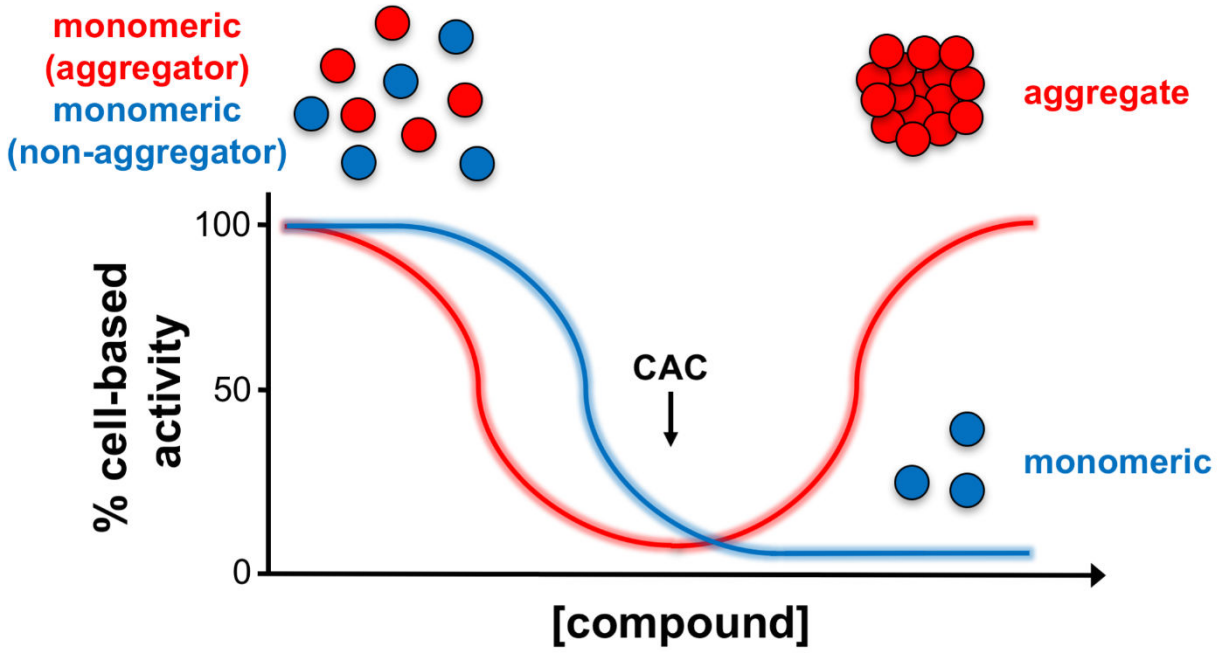


Figure 4. Cellular CRCs for aggregators. Some aggregators can exhibit bell-shaped ("U-shaped") CRC in cell-based assays. At low compound concentrations (below the CAC), monomeric compound is free to interact with the cellular components. Bioactive monomeric compounds will typically exhibit a standard dose-response. However, for aggregators at higher compound concentrations (at or above the CAC), the majority of compound forms an aggregate in the culture emedia (colloid), effectively sequestering bioactive compound and introducing aggregate-cell interactions.

Table 2. Suggested commercially available aggregation control compounds. Most of these compounds should form aggregates in the low micromolar range (e.g., 1-50 μM), though exact CACs will depend on experimental conditions including buffer composition. Additional examples can also be found in several seminal manuscripts (4,9,10,15,24). Additional examples of potential aggregation controls can be found online at PubChem (see Table 3 for relevant PubChem AIDs) and Aggregator Advisor.

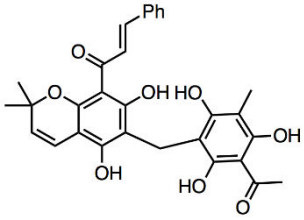
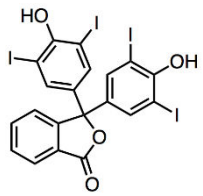
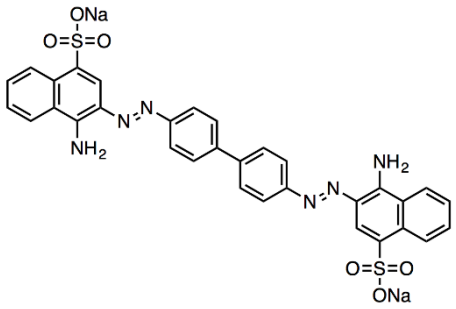
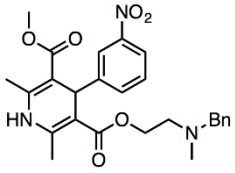
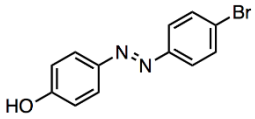
Compound	Chemical structure	Commercially availability	References
Rottlerin		Yes	(9)
TIPT		Yes	(9)

Table 2. continued from previous page.

Compound	Chemical structure	Commercially availability	References
Congo red		Yes	(9)
Nicardipine		Yes	(4)
4BPAP		Yes	(17)

Section Summary

Thoughtful assay design can potentially reduce the impact of nonspecific aggregation. Suggested strategies include the addition of detergents and decoy proteins, and careful consideration of enzyme and test compound concentrations. Assays can also be challenged with previously characterized aggregators to gauge the potential effect(s) of aggregation on assay readouts.

Strategies to Identify Aggregation

Introduction

Even with the aforementioned strategies in place, it is still possible to encounter aggregation. Alternatively, one may wish to investigate aggregation in previously identified bioactive compounds. This section describes several counter-screens and experimental strategies to identify aggregation among bioactive compounds. Primary methods to identify aggregators are discussed, including enzymatic counter-screens, CRC analyses, assay component titrations, and dynamic light scattering (DLS). Secondary methods to identify aggregation such as such as SPR, structure-interference relationships (SIR) , nuclear magnetic resonance (NMR), and microscopy are also discussed.

β-Lactamase Counter-Screen

The most popular and well-characterized counter-screen for aggregation is an AmpC β-lactamase enzymatic assay (25). The reasons for utilizing this particular enzyme are partly historic, as much of the published characterization of aggregation stems from an HTS targeting AmpC (9). In this assay, compounds are tested for inhibition of the *Escherichia coli* AmpC β-lactamase in the presence and absence of detergent (**Figure 5**). Most compounds should not show activity versus this target, regardless of detergent status, and non-aggregating, well-behaved inhibitors will show detergent-independent enzymatic inhibition. By contrast, aggregators will in many cases show detergent-dependent activity at low micromolar compound concentrations.

It is highly recommended to perform some variation of the AmpC assay as an aggregation counter-screen. This assay has several favorable characteristics: (a) it is highly robust; (b) it requires no specialized instrumentation aside from a standard absorbance plate reader; (c) the enzyme is relatively easy to produce and is also commercially available; and (d) there is an abundance of previously characterized control compounds.

Sample Steps | AmpC Counter-Screen

1. Prepare compound solutions. For testing compounds at 10 µM final concentrations, we prepare 500 µM working solutions for compatibility with the following assay protocol.
2. Prepare nitrocefin reagent stock solution. We prepare this solution as 5 mM nitrocefin in neat DMSO. Protect this solution from light. Stock solution is typically stable to multiple freeze-thaw cycles.
3. Prepare assay buffers. First prepare 10X stock solution without detergent. Then prepare two 1X buffer solutions (one without detergent, one with detergent).
4. Prepare enzyme stock solution. Prepare as 30X solution in assay buffer plus 0.0006% Triton X-100 (v/v). The minor amount of detergent prevents nonspecific enzyme adsorption to container walls.
5. Add 142 µL buffer to plate wells with multichannel pipette. We typically use 96-well plates (e.g., Corning Half-Area UV-transparent plates).
6. Add 5 µL of 30X enzyme solution to plate wells with multichannel pipette.
7. Add 3 µL of compound stock solution to plate wells with pipette. To do this step with a multichannel pipette requires "pre-plating" compound stocks in a separate microplate.
8. Mix by gently pipetting up and down three times. Ensure adequate mixing, as the neat DMSO has a tendency to pool at the bottom of plate wells if not sufficiently mixed.
9. Incubate solution from Step 8 for 5 min at room temperature.
10. Add 3 µL of nitrocefin reagent solution to solution from Step 9.
11. Mix by gently pipetting up and down three times. Ensure adequate mixing, as the neat DMSO has a tendency to pool at the bottom of plate wells if not sufficiently mixed.
12. Monitor reaction at 482 nm for 5 min using plate reader. Begin monitoring reaction as quickly as possible to best capture V_{max}.
13. Repeat Steps 5 through 12, substituting detergent-free buffer with detergent-containing buffer.
14. Analyze enzymatic activity using V_{max}.
15. Normalize percent activity to vehicle control (e.g., DMSO-only).
16. Compare activities between the detergent-free and detergent-containing reactions. Compounds are flagged as punitive aggregators if there is a significant attenuation* of bioactivity with the inclusion of detergent.
    * *Based on historical data in our lab, this is defined as > 20% reduction in activity. Other criteria can include > 3 SD from the negative control (untreated) wells.*

Researchers should consider the following technical points:

- The above protocol is an adaption of a previously published protocol (25).
- For general applicability, this protocol is based on multichannel pipettes rather than liquid-handling instruments.
- Compounds are typically tested at 10 µM final compound concentrations. However, it may be necessary to test at alternate concentrations, specifically if the CAC in the relevant assay system is potentially higher than 10 µM.
- Always include positive and negative control compounds. For suggested positive aggregation controls, refer to "Controls/reference compounds" section. We have historically used compounds such as lidocaine as negative aggregation control compounds.
- Recombinant *E. coli* AmpC can be prepared in-house, or purchased from a commercial source.
- Note different species of AmpC may have different susceptibilities to aggregation.

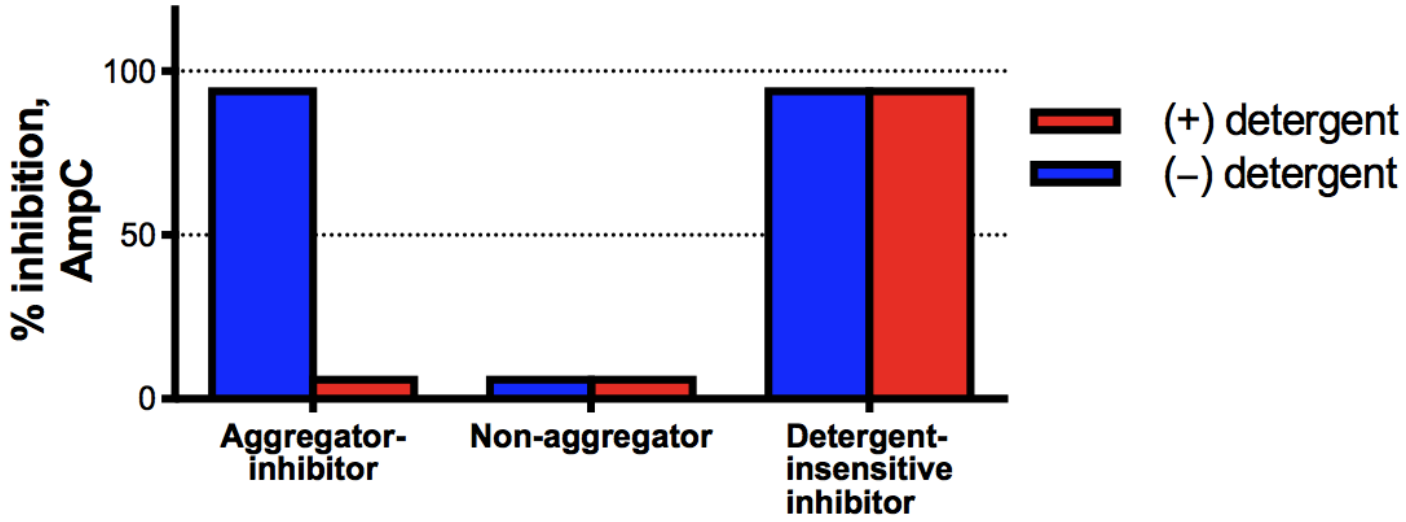


Figure 5. Representation of AmpC counter-screen for aggregation. Compounds are tested for inhibition of the AmpC B-lactamase in the absence (blue) and presence (red) of 0.01% Triton X-100 (v/v). Putative aggregators are most often identified by a significant attenuation of activity upon the inclusion of detergent (while not shown, some aggregators can activate AmpC activity). Non-aggregators typically show minimal activity in both the presence and absence of detergent. Detergent-insensitive inhibitors, which can include well-behaved compounds, show similar activity in the presence and absence of detergent.

- Purification tags may also alter the behavior of a protein with respect to aggregation.
- Different reaction buffers can likely be used for this counter-screen. For example, one may wish to test in the exact buffer as the original HTS. Always verify enzyme activity in any new buffer system.
- Large bubbles can be removed by brief centrifugation or manually by gentle blowing. Excessive bubbling often results from vigorous/turbulent dispensing and mixing, as well as excess detergent (10). To reduce bubbling, mixing should occur below the liquid level of the reaction well (25).
- Prepare detergent solutions fresh for each experiment, as Triton X-100 can produce H_2O_2 in aqueous solution (14).
- Pure detergents can take several minutes to an hour to completely dissolve when transferred to aqueous solutions. Verify detergent is completely dissolved before proceeding with experiments.
- Pure detergents can be highly viscous and difficult to pipette. Prepare 10% solution (v/v) in H_2O for easier, more precise dispensing.
- Depending on the enzyme source, purity, and method of protein quantification, a necessary experiment is to titrate the concentration of enzyme.
- Keep enzyme solutions on ice when not in use during the course of an experiment.

Detergent Titration Counter-Screen

As compound aggregates can have varying sensitivities to detergents and also different CACs, another tactic to identify aggregates is to perform a series of detergent titrations in the original assay system. Non-aggregating compounds will have minimal changes in bioactivity with increasing concentrations of detergent. By contrast, aggregates may demonstrate decreased potencies with increased concentrations of detergent (**Figure 6**).

Sample steps | Detergent Titration Counter-Screen

1. Prepare two or more buffer solutions: one containing no detergent, the other(s) containing varying levels of detergent (e.g., 1X, 5X, 10X)
2. Prepare parallel reaction solutions using the detergent and detergent-free buffers.

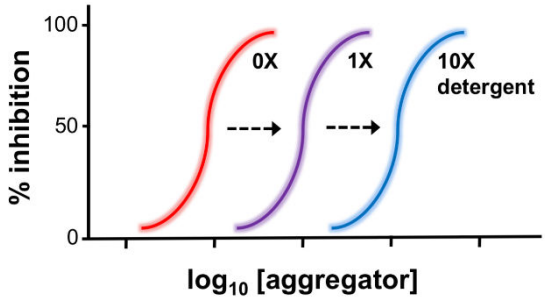


Figure 6. Representation of a detergent titration experiment to identify aggregation. For aggregators, the IC_{50} values can increase with increasing amounts of detergent. Shown is a hypothetical inhibitory aggregator (n.b. steep Hill slope).

3. Test compound in the detergent-free buffers.
4. Test compound in the detergent-containing buffers.
5. Compare activity in the presence and absence of detergent.

Researchers should consider the following technical points:

- Verify the target protein(s), test compound(s), assay reagent(s), and assay readout tolerate the specific detergent being used in this counter-screen.
- Because it can prevent protein adsorption to container walls, detergent-containing reactions may have more active enzyme, and consequently, may have greater overall bioactivity than otherwise identical detergent-free reactions.
- Aggregation is suspected when bioactivity is significantly attenuated by the inclusion of detergent. The exact magnitude of this effect will depend on the specific target and assay system. A rule of thumb is rightward shifts in IC_{50} > 3X should be considered likely aggregate-dependent effects and further analysis with higher detergent concentrations may be warranted if the compound is not immediately eliminated from further follow-up consideration. Depending on assay precision, IC_{50} values can fluctuate, and a useful tool for assessing the significance of changes in IC_{50} values is the minimum significant ratio (MSR). For detailed discussion, the reader is referred to Minimum Significant Ratio – A Statistic to Assess Assay Variability.

Hill-Slope Analysis

A salient feature of aggregation are steep CRCs (**Figure 7**) (4,10,19,27). This reflects the CAC, the high affinity of the protein-aggregate complex, and the protein-compound stoichiometry. Based on the Hill Equation (**Equation 2**), the "steepness" of a given CRC is quantified by Hill coefficient ("Hill slope").

$$\varnothing = \frac{[\mathrm{L}]^n}{K_d + [\mathrm{L}]^n} \qquad \text{Equation 2}$$

(where ∅ represents fraction of receptor bound to ligand, [L] represents free (unbound) ligand concentration, K_d represents apparent dissociation constant, n represents Hill coefficient)

For example, a well-behaved inhibitor with non-cooperative binding will have a Hill slope of 1.0, with approximately 90% of the bioactivity spanning across two orders of magnitude. In general, many aggregators will have Hill slopes greater than 1.5, some even being greater than 5.0. As a word of caution, a subset of aggregators can also have Hill slopes close to 1.0.

Calculating Hill slopes for CRC data is performed by regression analyses. Many labs will use commercial software packages such as GraphPad Prism. Many default curve-fitting algorithms will assume a fixed slope (Hill

slope equal to 1.0), so it is critical to select a "variable slope" curve-fit. For example, a sigmoidal CRC is often fit to a four-parameter variable slope equation (**Equation 3**) (28, 29).

$$Y = \frac{Low + (High - Low)}{1 + 10^{(\log EC_{50} - X)(Hil\ slope)}} \quad \text{Equation 3}$$

(where Y represents assay activity, Low and High represent the low and high bounds)

Depending on the shape and completeness of the CRC, additional constraints may need to be specified for an accurate curve-fit. For additional details on calculating the Hill slope, refer to Assay Operations for SAR Support.

There are some caveats to using Hill slope analysis for aggregation. First, aggregators with Hill slopes close to 1.0 have been described (10). Second, covalent modifiers can also have steep Hill slopes (10). Third, as mentioned previously, any situation where [E] >> K_d of the inhibitor will result in a steep Hill slope.

Enzyme Concentration Titration

Another relatively straightforward approach to identify nonspecific inhibition by aggregators is to perform a series of enzyme titration experiments (19,25). Based on the aforementioned stoichiometric model of aggregation inhibition, the IC_{50} value of an aggregator is expected to increase linearly with increases in enzyme concentration (for single-point experiments, percent inhibition should decrease when the concentration of enzyme is increased).

Sample Steps | Enzyme Titration Counter-Screen

1. Test compound at standard enzyme concentration (i.e., 1X) under standard conditions. Also perform vehicle control in parallel.
2. Determine activity of test compound from Step 1 relative to vehicle control.
3. Test same compound from Step 1, except at 5X enzyme concentration under otherwise standard conditions. Also perform vehicle control in parallel with this same enzyme concentration.
4. Determine activity of test compound from Step 3 relative to vehicle control.
5. Repeat Steps 3 and 4 with increasing concentrations of enzyme (e.g., 10X, 20X)
6. Plot IC_{50} versus enzyme concentration. A linear relationship is suggestive of stoichiometric inhibition, consistent with aggregation.

Researchers should consider the following technical points:

- If available, include a non-aggregator positive control compound. The activity of such a compound should not proportionally increase with additional enzyme (unless enzyme concentration significantly exceeds the K_d value of the inhibitor).
- Verify the effect of increased enzyme on reaction progress. With increased enzyme, reactions will proceed quicker and not approximate steady-state conditions due to increased product formation. Also verify assay readout is within the dynamic range of the detection system.

Centrifugation Counter-Screen

Aggregators will often show a pronounced decrease in bioactivity when reaction solutions are centrifuged (4). This process effectively pellets the aggregates, leaving only monomeric compound (and non-sequestered protein if present) in the supernatant (**Figure 8**). Notably, this counter-screen does not identify potential aggregators based on differential bioactivity in the presence and absence of detergents. For this reason, *this particular counter-screen may be especially useful in assay systems intolerant to detergents or decoy proteins.*

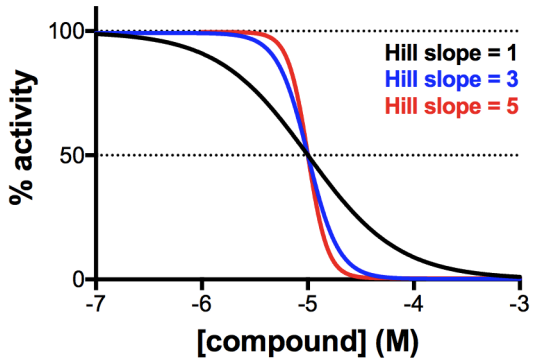


Figure 7. Hill slope analysis to identify aggregation. Shown is a simulation of compounds with identical 10 µM IC_{50} values. Aggregators characteristically have steep CRCs. A well-behaved compound (black line) typically has a Hill slope equal to 1.0, whereas many aggregators have higher Hill slopes (blue, red lines).

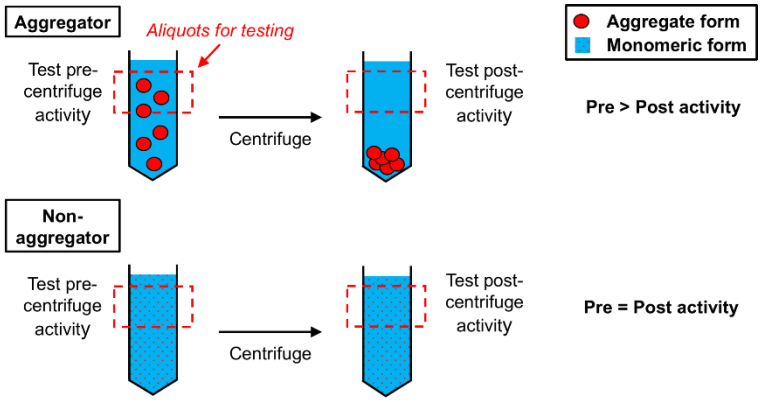


Figure 8. Centrifuge-based experiment to identify aggregation. Aggregates (top panel) can usually be removed by brief centrifugation of samples. For aggregators, post-centrifugation supernatant aliquots should show reduced bioactivity compared to pre-centrifugation samples. By contrast, non-aggregators (bottom panel) should have similar activity pre- and post-centrifugation.

Sample Steps | Centrifugation Counter-Screen

1. Incubate test compound in assay buffer for 5 min.
2. Remove supernatant aliquot from Step 1 for testing.
3. Centrifuge mixture from Step 1 at approximately 15,000 g for 30 min at 4 °C. Potential aggregates should form a sediment.
4. Remove supernatant aliquot from Step 3 for testing.
5. Test and compare bioactivity of aliquots from Steps 2 and 4 by adding remaining assay reagents (e.g., target enzyme(s), substrate(s), co-factor(s), etc.).

Researchers should consider the following technical points:

- Include a vehicle-only control (e.g., DMSO-only) to control for changes in enzyme activity during the centrifugation process.
- Also include a known non-aggregating positive control compound (if known).
- Both types of controls should be tested for bioactivity using pre- and post-centrifuged aliquots.
- For poorly soluble compounds, also consider simple precipitation of compound rather than colloid formation.

- As a control for the centrifugation process, store the pre-centrifugation aliquots in conditions similar to the centrifugation (e.g., 4 °C).

Pre-incubation Counter-Screen

A simple mechanistic counter-screen for suspected aggregators is to modify the compound-protein pre-incubation time. Aggregators will often show more potent activity when allowed to pre-incubate with the target protein (4,9). In an AmpC assay, the magnitude of this effect varied from several-fold to greater than 50-fold changes in IC_{50} values (9).

Notably, this counter-screen does not identify potential aggregators based on differential bioactivity in the presence and absence of detergents. For this reason, *this particular counter-screen may be useful in assay systems intolerant to detergents or decoy proteins, or to follow-up on those compound activities that may fall into the aggregator detergent-insensitive class* (see **Figure 4**).

The counter-screen premise is relative simple. For a conventional enzymatic assay, compounds are tested in two different reaction systems: (1) compounds and proteins are pre-incubated together for 5 min, then the reaction is initiated by addition of non-proteinaceous substrate, and (2) compounds and non-proteinaceous substrate are incubated together, then the reaction is initiated by addition of protein. In the first reaction, when aggregates and protein are allowed to incubate together for even 5 min, most of the protein will adsorb to the aggregates (in the absence of other strategies to mitigate aggregation such as detergents and/or decoy protein). In the second reaction, no such adsorption occurs between the non-proteinaceous assay components and aggregates when incubated together. When this second reaction is initiated by the addition of protein, there is a higher initial concentration of unbound (and unmodulated) protein.

This tactic is generally best when performed as a counter-screen, rather than in the HTS phase where one may consider reducing pre-incubation times to reduce assay interference from potential aggregation. Reducing pre-incubation times is difficult in many HTS settings due to instrumentation and scale. Furthermore, pre-incubation is often purposefully designed to allow for equilibration purposes.

1. Incubate test compound and protein for 5 min. Also perform vehicle control.
2. Initiate reaction by adding non-proteinaceous substrate to mixture from Step 1.
3. Determine activity from reaction from Step 2 relative to vehicle control.
4. In a separate reaction, incubate test compound with non-proteinaceous assay components for 5 min. Also perform vehicle control.
5. Initiate reaction by adding proteins (e.g., target enzyme) to mixture from Step 4.
6. Determine activity from reaction from Step 5 relative to vehicle control.
7. Compare activities from Steps 3 and 6.

Researchers should consider the following technical points:

- Include a known non-aggregating positive control compound (if known).
- The suggested length of pre-incubation is 5 min, though it is possible this interval may need to be extended for slower-forming aggregate-protein complexes.
- Recall the differential activity of aggregators will depend on the formation of aggregates during the pre-incubation phase. If sufficient amounts of detergent to dissolve aggregates are already present in the assay, minimal differential activity may be observed.
- For compounds showing increased bioactivity with protein pre-incubation, one should also consider the possibility of irreversible, time-dependent target modulation. Refer to Assay Interference by Chemical Reactivity for additional details on the identification of reactivity.

Dynamic Light Scattering

Dynamic light scattering (DLS) is a biophysical technique used to determine particle sizes in solution. Compounds are dissolved in solution, and if aggregation occurs, the size of aggregates can be quantified by measuring temporal fluctuations in light scattering and generating an autocorrelation function (**Figure 9**). There are several examples of DLS used in the identification and characterization of aggregates (2,4,6,24,30). Modern instruments utilize either cuvettes or microplates, the latter being amenable to higher-throughput counter-screens. This technique provides direct, physical evidence of aggregate formation, though it does not provide functional information about any detected aggregates. Another advantage is that the assay is not dependent on the particular target (protein-free), and in theory it can be adapted to model assay specific buffer conditions. Depending on available instrumentation and desired throughput, DLS can be a highly useful primary counter-screen for aggregation.

Structure-Interference Relationships

Structure-activity relationships (SAR) can often be explained by assay interference, so-called structure-interference relationships (SIR). Analysis for SIR in the context of HTS can help identify potential aggregators by analyzing specific bioactive chemotypes in-depth. This approach can be especially helpful for equivocal analyses, where it is unclear whether a bioactive chemotype is a function of aggregation or useful target engagement. If the apparent SAR appears to correlate with aggregation, then it can facilitate triage of related analogs and also inform the design of related chemotypes to prevent the formation of colloids. As with conventional SAR analyses, this approach is most helpful with multiple compounds. Bioactivity analysis should focus not just on the active compounds and close analogs, but also on structurally-related inactive compounds. If a chemotype is particularly important for a project, this analysis can be aided by SAR-by-commerce, in which closely related analogs (active and inactive) can be purchased and tested for activity in the original assay and aggregation counter-screens.

This approach was particularly helpful in the post-HTS triage of a 3-hydroxy-pyrrolidin-2-one core active against the fungal histone acetyltransferase (HAT) Rtt109 (**Figure 10**). In this case, clues that an apparent SAR actually reflected an aggregation-dependent SIR included: (a) little change in activity with minor aromatic substituent changes, (b) correlation of bioactivity with increased lipophilicity and molecular weight, and (c) a precipitous drop in activity with the removal of the *C3*-hydroxy group. Of course, these findings could also be explained by true bioactivity, which should also be considered in any such analysis. In this example the correct identification of aggregation as the primary source of bioactivity ultimately depended on correlation with other experimental data (AmpC counter-screen, steep Hill slopes, detergent titration, mechanism of action studies) (31). By determining the specific components contributing to aggregation, there was high confidence in discontinuing work on this chemotype.

Computational Approaches

Cheminformatics can also be utilized to help identify potential aggregators, especially in the context of the large datasets inherent to HTS. Since the factors contributing to aggregation are multifactorial, most current cheminformatics tools for aggregation triage are based on chemical similarity to known aggregators.

A helpful, open-resource for flagging potential aggregators is Aggregator Advisor (32). In this method, compound structures in the form of SMILES can be submitted to a database of known aggregators. Compounds with a high-degree of similarity to known aggregators are flagged. This page also contains an extensive list of aggregators ("Rogues' Gallery").

Datasets from several HTS campaigns in the presence and absence of detergents are also publically available *via* PubChem, which can facilitate additional data analyses and method development (**Table 3**).

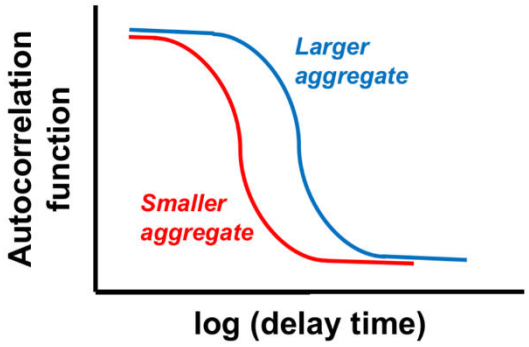


Figure 9. Dynamic light scattering for the characterization of aggregates. Due to their sizes, aggregates will scatter light. The size of aggregates can be characterized by DLS by plotting the autocorrelation function as a function of delay time. Larger aggregates will maintain a higher correlation for longer delay times relative to smaller aggregates (compare blue and red curves).

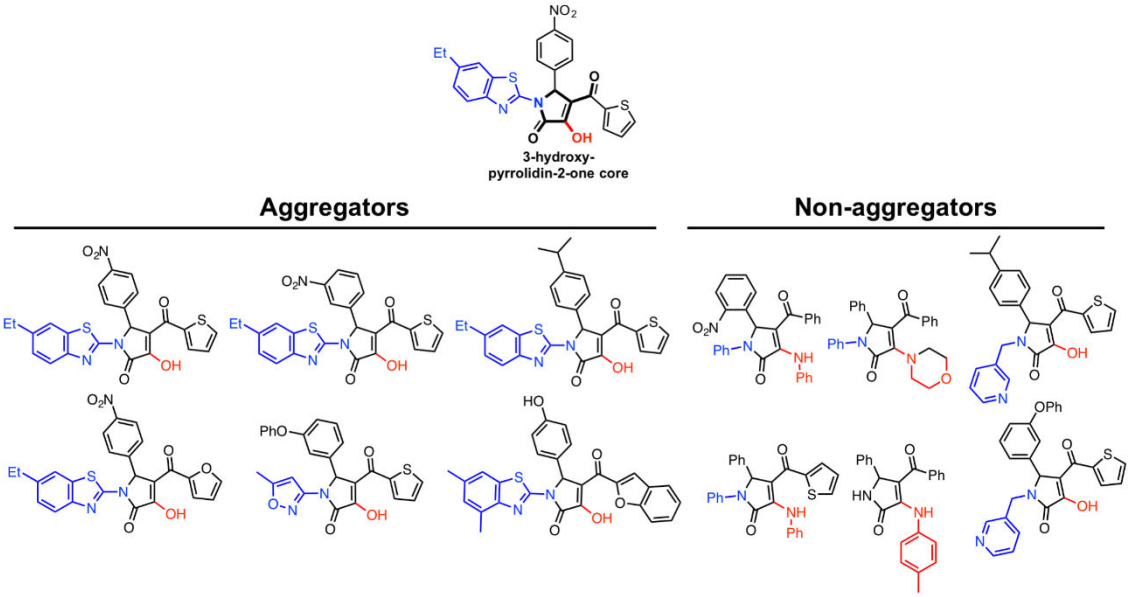


Figure 10. Structure-interference relationships of aggregators. Analysis of analogs can help identify bioactivity due to aggregation. In this example from an HTS for inhibitors of yeast Rtt10930, most compounds with the 3-hydroxy-pyrrolidin-2-one core are active at low micromolar compound concentrations versus Rtt109, and are also flagged as aggregators by the AmpC aggregation counter-screen. Compounds without the *C3*-hydroxy group were inactive versus Rtt109 and inactive in the AmpC counter-screen (*C3* substituents highlighted in red). Compounds with alkyl substituents at the *N1*-position were also inactive versus Rtt109 and inactive in the AmpC counter-screen (*N1* substituents highlighted in blue).

Table 3. PubChem HTS data relevant to aggregation.

PubChem Assay	PubChem Assay ID
qHTS Inhibitors of AmpC Beta-Lactamase (assay without detergent)	485341
qHTS Inhibitors of AmpC Beta-Lactamase (assay with detergent)	485294
qHTS Assay for Promiscuous and Specific Inhibitors of Cruzain (without detergent)	1476
qHTS Assay for Promiscuous and Specific Inhibitors of Cruzain (with detergent)	1478
Promiscuous and Specific Inhibitors of AmpC Beta-Lactamase (assay without detergent)	585
Promiscuous and Specific Inhibitors of AmpC Beta-Lactamase (assay with detergent)	584

Nuclear Magnetic Resonance

Two different nuclear magnetic resonance (NMR) techniques have been described to identify aggregates, one protein-free ("ligand-based") and the other utilizing a protein probe ("protein-based").

In one counter-screen, standard proton NMR can be used to identify aggregates without the need for target protein (33). Compared to monomeric (non-aggregating) forms, aggregates can exhibit characteristic chemical shift patterns due to the additional intermolecular effects from compounds in close proximity. These chemical shift perturbations revert to the monomeric chemical shift patterns upon dilution, with the addition of detergent, or after centrifugation (**Figure 11**). These chemical shift patterns include chemical shift change(s), additional peak(s), and change(s) in peak shape. The advantages of this technique include its lack of required protein, its use of relatively standard instrumentation (i.e., proton NMR), and a biophysical readout. The main disadvantage of this assay is its comparatively lower throughput. Like DLS and microscopy, it can detect aggregation but does not inform the functional consequence of these aggregates on bioactivity. For details on performing this counter-screen, see the original manuscript for an excellent experimental walkthrough (33).

Another technique, a La assay to detect reactive molecules by nuclear magnetic resonance (ALARM NMR), is sensitive to nonspecific protein perturbation by known aggregators (31). This assay is an industry-developed [^{1}H-^{13}C]-HMQC counter-screen originally developed to identify nonspecific thiol-reactive screening compounds by their ability to induce DTT-dependent conformational shifts in the La antigen (34,35). As they exert their bioactivity through non-covalent interactions, aggregators can perturb the La antigen conformation independent of DTT (**Figure 12**). An advantage of using ALARM NMR is its ability to identify nonspecific thiol-reactivity *and* aggregation. However, potential drawbacks of this method are the specialized instrumentation required, its medium throughput (approximately 50 samples per day), and the relatively high concentrations of compound required for testing (i.e., mid-micromolar). As the La antigen may not be sensitive to every aggregator (JL Dahlin, unpublished observations) and the experimental conditions may not approximate the relevant original screening conditions, we recommend following up critical results with additional counter-screens.

Surface Plasmon Resonance

Another biophysical technique for detecting aggregation utilizes surface plasmon resonance (SPR) biosensors. Aggregates can produce characteristic readouts in SPR experiments, most notably superstoichiometric binding (**Figure 13**) (8). An interesting finding from this original study is that the nature of any given aggregate-protein interactions can vary depending on the particular compound *or* the target protein identity. This technique can capture multiple modes of aggregation-target interactions, including stoichiometry and the kinetics of aggregate association and dissociation. Like many of the other aforementioned aggregation counter-screens, aggregation in this SPR setup is generally attenuated by the addition of detergent. The advantages of this technique include its general applicability to any target amenable to SPR, and its biophysical readout, which provides information about the kinetics of direct aggregate-protein interactions. The main disadvantages of this technique are its instrumentation requirements (i.e., non-standard in many academic labs), need for protein immobilization ("on chip"), and overall throughput. For details on performing this counter-screen, we refer to the original manuscript for several example aggregation readouts (8).

Microscopy

While not a routine tool due to the specific instrumentation required and the relatively low throughput, aggregates can also be directly visualized through specialized microscopy. The most common microscopy method for observing aggregates is transmission electron microscopy (TEM) (4,9,36,37). A general procedure involves preparing the compound aggregate in assay buffer, then transferring to a carbon-coated grid, followed by negative staining with uranyl acetate or ammonium molybdate. Aggregates can also be indirectly visualized

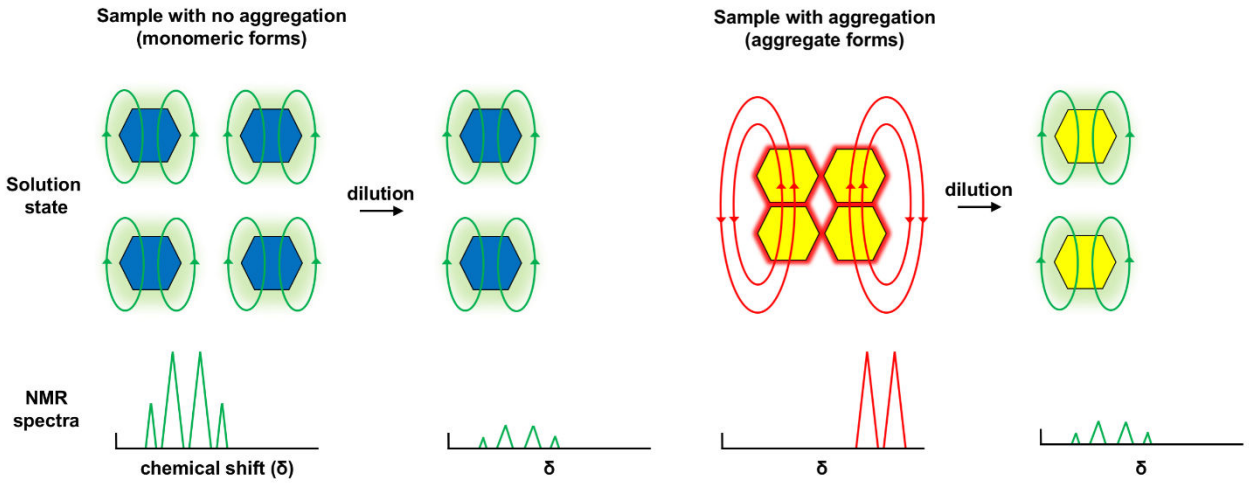


Figure 11. NMR-based detection of aggregation. (Left) Non-aggregating compounds (blue) exist in monomeric form, producing characteristic chemical shifts (depicted as green lines). Dilution of the sample does not produce chemical shifts, just dilution of signal intensity. (Right) Aggregating compounds (yellow) will exist as aggregates, producing a set of chemical shifts specific for the aggregate form (red lines). Dilution of the sample below the CAC disrupts the aggregate to form monomeric compound, which have a different chemical shift pattern (green lines).

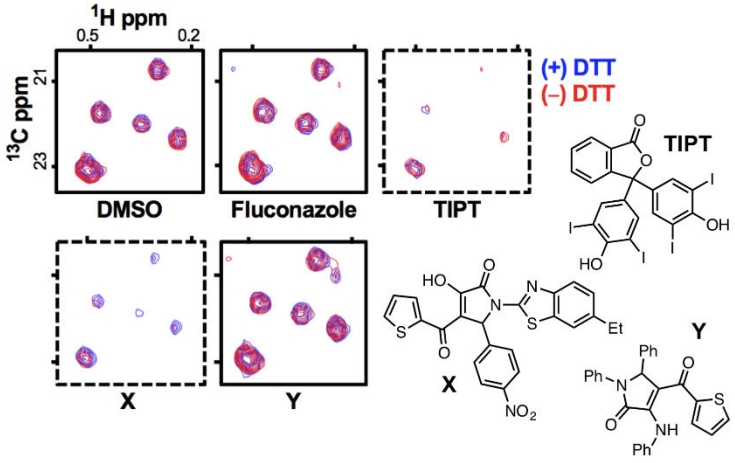


Figure 12. ALARM NMR readout sensitive to aggregation. Known aggregators TIPT and compound **X** perturb the La antigen conformation independent of DTT (9,31). Compound **Y**, a structural analog of **X**, is used as an aggregation negative control compound. Fluconazole included as additional non-reactive, non-aggregation control compound. Dashed border, ALARM NMR-positive.

with fluorescent microscopy when incubated with fluorescent proteins, which can adsorb to the surface of aggregates (4). The direct observation of adducts should be correlated with additional bioactivity and mechanistic data.

Section Summary

Bioactivity due to nonspecific aggregation can be identified through several strategies. Initial tests to evaluate for aggregation typically include enzymatic counter-screens, CRC analyses, DLS, and simple mechanistic experiments. More advanced and/or resource-intensive methods include SPR, NMR, and microscopy. In addition to triaging nonspecific compounds, these strategies can also be useful for de-risking compounds as they proceed along the discovery and development pipeline.

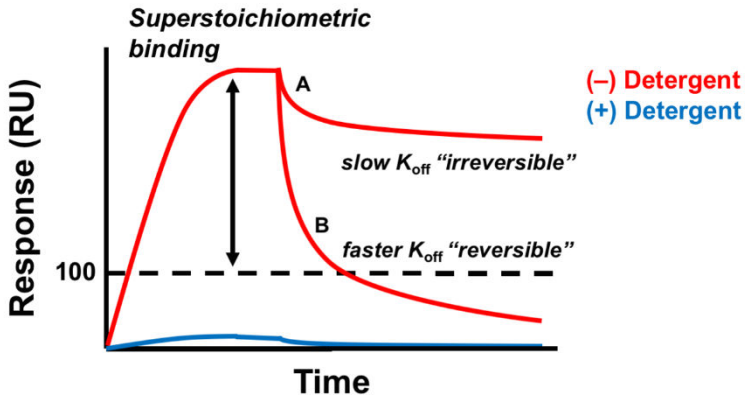


Figure 13. SPR characterization of aggregators. Shown is a simulation of a prototypical aggregator in a standard SPR experiment. In this example, response is normalized to a well-behaved inhibitor that binds the protein in a 1:1 ratio (RU = 100). Aggregators can produce superstoichiometric binding (characterized by large responses) and can display detergent-dependent protein binding (compare red and blue lines). Aggregates can display either slow dissociation mimicking irreversible binding (curve A) or faster dissociation (curve B).

Frequently Asked Questions

This section seeks to answer some common questions about aggregation in the context of drug and chemical probe discovery, including some common misconceptions (adapted from advisor.bkslab.org).

1 **Can aggregation affect cell-based assay readouts?**

Yes. There are several examples of aggregators modulating cell-based readouts. Interference and nonspecific bioactivity can result from direct interaction with membrane-bound receptors (38), a reduction in bioactive monomeric compound (37), or interactions with other extracellular factors (36). Aggregators have also been associated with cytotoxicity and changes in cellular histone acetylation (JL Dahlin *et al.*, forthcoming results). It is unclear if aggregates can form intracellularly. Studies with Evans blue suggest aggregates do not transverse individual cell membranes intact (e.g., by endocytosis) (22). Some known aggregators exhibit U-shaped curves due to aggregation at high compound concentrations which effectively prevents cell penetration (22). Other studies with Evans blue suggest certain aggregates may cross certain tissue structures such as tumor blood vessels via increased permeability (37). Compounds can potentially form aggregates *in vivo*, based on simulated intestinal fluid studies (39). Therefore, it is highly advised to consider the use of aggregator controls in cell-based assays, especially when testing compounds at micromolar concentrations. It is currently unclear which strategies, including the use of detergents and decoy proteins, can most effectively mitigate aggregation in cell-based experiments, especially without perturbing cell function.

2. **Several FDA-approved drugs are aggregators. How can this observation be reconciled with the recommendation to triage aggregators?**

Several drugs can form aggregates in enzymatic assays at micromolar concentrations (24). However, many of these drugs have been extensively optimized for potency and selectivity, and are typically used at nanomolar compound concentrations, well below their CACs.

3. **Can one predict aggregation based on chemical structure (e.g., *in silico*)?**

Several methods have been described to flag potential aggregators based on structure and other properties (24,30,32,40,41). As with any model, there is always the potential for false-positive and false-negative

predictions. While helpful for economically analyzing large numbers of compounds, these tools should not be used as sole substitutes for well-designed experimental counter-screens for aggregation.

4. **Do aggregators always inhibit enzyme activity?**

No. While the most common effect of partial protein unfolding by aggregation in enzymatic assays is inhibitory, it is possible to actually enhance target activity (10). For example, we have observed several reproducible cases of reported HAT inhibitors actually increasing AmpC activity (JL Dahlin *et al.*, forthcoming results). The mechanistic basis of this phenomenon is unclear, but it may be influenced by reaction volume and/or compound solubility.

5. **Can a compound aggregate in all assay conditions?**

No. Aggregation (i.e., CAC) depends on multiple factors, including assay buffer components, ionic strength, pH, and detergent (42). Other experimental factors such as pre-incubation time, temperature, mixing procedures, and organic solvents (e.g., DMSO) may also alter aggregation behavior. These factors, combined with the actual compound concentration, determine whether a compound aggregates or not.

6. **Do aggregators always show a complete concentration-response?**

Not necessarily. Aggregator CRCs can plateau before achieving a complete dose-response due to compound precipitation (19).

7. **What other HTS phenomenon can be attributed to aggregators?**

Gain-of-signal outputs have been observed, possibly the result of an aggregation phenomenon. In one case, an HTS TR-FRET assay identified a very large number of primary actives (a compound-dependent positive FRET signal). Given it was unlikely that a productive protein-protein interaction would be facilitated by these compounds, the possibility exists this effect was caused under a situation where aggregates created a surface upon which donor and acceptor labeled proteins adhered and resulted in a non-mechanism relevant proximity, thereby producing the positive FRET signal (J Inglese, personal observation ca. 2003).

Additionally, there is also the possibility for aggregates to interfere with antibody-based assays by sequestering immunoglobulin reagents.

Conclusions

Aggregation represents a significant source of compound-mediated assay interference and nonspecific bioactivity in biological assays which utilize small molecules. The incidence of aggregation by test compounds can usually be mitigated by careful assay design, such as the use of detergents and the optimization of enzyme and test compound concentrations. Aggregators can be identified by several strategies (**Table 4**), including enzymatic counter-screens, DLS, and various titration experiments. Cheminformatics and advanced strategies such as microscopy, NMR, and SPR can also identify aggregators.

Several recommendations are made to prevent wasted follow-up on aggregators in the context of HTS, chemical biology, and related fields.

1. Proactively design assays to mitigate the effect and the prevalence of aggregation.
2. Empirically test bioactive compounds for aggregation by at least one method (i.e., test regardless of whether aggregation is suspected or not).
3. Compounds with a high index of suspicion for aggregation should be evaluated by multiple methods.
4. Testing for aggregation should attempt to approximate the relevant biological assay conditions.

Following these strategies should triage intractable chemical matter and de-risk more promising bioactive compounds.

Table 4. Summary of aggregation counter-screens.

Assay	Pros	Cons	Notes
AmpC β-Lactamase	-Well-characterized -Robust -Medium-to-high throughput -Requires relatively standard instrumentation (i.e., plate reader)	-AmpC assay conditions might not be compatible with target assay conditions -Not all aggregators modulate AmpC	-AmpC commercially available -Provides additional information about target specificity -Assay subject to other forms of compound-mediated interference -Indirect evidence of aggregation
Detergent titration	-Utilizes original assay target and assay technique -Assays target modulation by aggregates	Detergents might not be compatible with target assay conditions	-Assay subject to other forms of compound-mediated interference -Indirect evidence of aggregation
Hill slope	Can analyze existing CRC data	Aggregators do not always exhibit steep Hill slopes	Indirect evidence of aggregation
Enzyme titration	-Utilizes original assay target and assay technique -Assays target modulation	May require significantly adjusting assay time points and other variables	-Assay subject to other forms of compound-mediated interference -Indirect evidence of aggregation
Centrifugation	-Utilizes original assay target and assay technique -Assays target modulation	High-force centrifugation generally lower throughput	Assay subject to other forms of compound-mediated interference
Cheminformatics	-Requires only chemical structure as input -High-throughput	-Training sets based on specific assay conditions, targets; applicability to other experimental variables unknown -Susceptible to false-positive and false-negative results	Experimental confirmation strongly recommended
DLS	-Biophysical evidence of aggregates -Medium throughput -Direct observation of aggregates	-Requires non-standard instrumentation -Does not assay target modulation	
NMR	-Biophysical evidence of aggregates -Does not require protein (ligand-based)	-Lower throughput -Does not assay target modulation -Requires non-standard instrumentation	Indirect evidence of aggregation
ALARM NMR	-Biophysical evidence of aggregates -Provides additional information about target specificity and nonspecific thiol reactivity	-Lower throughput -Requires relatively high protein and compound concentrations -Protein may not be universally susceptible to aggregates -Requires non-standard instrumentation	Indirect evidence of aggregates
SPR	-Biophysical evidence of aggregates -Can be adapted to target or surrogate proteins	-Lower throughput -Requires non-standard instrumentation -Does not assay target activity	Indirect evidence of aggregates

Table 4. continued from previous page.

Assay	Pros	Cons	Notes
Microscopy	-Lower throughput -Direct observation of aggregates	-Requires non-standard instrumentation -Does not assay target activity	

Suggested Web Resources

1 Aggregator Advisor (advisor.bkslab.org).

An excellent web-based cheminformatics resource for identifying potential aggregators. The input is test compounds in SMILES format, and the output is a similarity score relative to known aggregators.

2. PubChem (pubchem.ncbi.nlm.nih.gov).

A free resource for several HTS campaigns for aggregation.

Suggested Readings (alphabetical order)

1. Babaoglu, K.; Simeonov, A.; Irwin, J. J.; Nelson, M. E.; Feng, B.; Thomas, C. J.; Cancian, L.; Costi, M.P.; Maltby, D. A.; Jadhav, A.; Inglese, J.; Austin, C. P.; Shoichet, B. K. *J Med Chem*, **2008**, *51*, 2505.

   A detailed follow-up mechanistic analysis of aggregators from a biochemical HTS campaign (accompanies Suggested Reading #3).

2. Feng, B. Y.; Shoichet, B. K. *Nat Protoc*, **2006**, *1*, 550.

   An easy to follow step-by-step guide describing the AmpC β-lactamase counter-screen for aggregation.

3. Feng, B. Y.; Simeonov, A.; Jadhav, A.; Babaoglu, K.; Inglese, J.; Shoichet, B. K.; Austin, C. P. *J Med Chem*, **2007**, *50*, 2385.

   A representative example of the prevalence of aggregation in a biochemical HTS campaign, demonstrating ~95% of primary actives were detergent-sensitive aggregators (accompanies Suggested Reading #1).

4. McGovern, S. L.; Helfand, B. T; Feng, B.; Shoichet, B. K. *J Med Chem*, **2003**, *46*, 4265.

   An excellent discussion of the biochemical mechanisms of aggregation.

Acknowledgements

JLD acknowledges Dr. Michael A. Walters for helpful discussions and research support.

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