ACS MEASUREMENT SCIENCE Au
AN OPEN ACCESS JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

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Editorial

Underestimation of the Complexity of K_d Determination: Causes, Implications, and Ways to Improve

Cite This: ACS Meas. Sci. Au 2024, 4, 231−232
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The equilibrium dissociation constant (K_d) of biomolecular complexes is a fundamental physicochemical parameter frequently determined in various fields of scientific research. A quick search on Google Scholar reveals that at least 3,000 publications document thousands of K_d values each month. However, this number merely hints at the extensive scope of this area, as the bulk of K_d determinations are generally executed through highly parallelized high-throughput screening approaches within drug-discovery companies solely for internal use.

Numerous methods based on different physical principles, such as calorimetry, optical spectroscopy, physical separation, and biosensors have been developed for K_d determination. These techniques are widely integrated into various commercial instruments, making K_d determination remarkably user-friendly. Consequently, elucidating the K_d is often perceived by researchers as a trivial technical procedure involving the loading of reagents into instruments and reading of resulting K_d values. This trivialization of K_d determination has far-reaching implications.

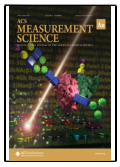
The random error reported along with the average K_d value indicates K_d precision but provides no information about K_d accuracy. A systematic error, a descriptor of accuracy, is defined as a difference between the determined value and the true value, the latter of which is fundamentally unknown, thus making accuracy assessment a vicious cycle. However, K_d determination is known to be prone to inaccuracies caused by large systematic errors, which can be mitigated through additional control experiments.[1] Yet, most publications reporting K_d values fail to mention such efforts, potentially resulting in grossly inaccurate data.[2] These inaccuracies can lead to both false-positive and false-negative conclusions. False-positive conclusions, stemming from underestimated K_d values, can misguide research efforts, while false-negative conclusions, arising from overestimated K_d values, can result in the overlooking of promising leads in pharmaceutical development. While false positives are often identified through attempts to replicate results,[3] false negatives are less likely to be detected. Overall, underestimating the complexity of K_d determination leads to inefficient allocation of research and development resources across various scientific fields as well as the drug-discovery industry.

It is worth noting that the oversimplification of K_d determination extends not only to users but also to method developers and instrument designers. There are numerous examples of methods and instruments utilizing nonadditive signals, which inevitably lead to systematic errors in K_d.[4] It is evident that the scientific community requires a comprehensive mitigation plan to address the inaccuracies associated with K_d determination. Such a plan should encompass efforts in both research and education.

Research efforts should aim to address the remaining knowledge gaps. Among these gaps is the lack of a method for quantitatively assessing the accuracy of determined K_d values. This vicious cycle of accuracy assessment could be broken if there were a reference standard for the determined value. While standards exist for length, time, charge, and mass, none exist for K_d, making the accuracy assessment of K_d an elusive goal. One potential avenue for addressing this goal is through the propagation of systematic errors. Classically, K_d values are computed by nonlinear regression from binding isotherms utilizing three variables: two concentrations and a fraction R of unbound (or bound) limited component. If systematic errors of concentrations and/or R can be assessed, then a systematic error of K_d can be propagated from them.[2] The direct link between the systemic error of K_d and those of concentrations and R underscores the need to find ways to estimate systematic errors of concentrations and R. If accomplished, an approach could potentially be developed for the assessment of systematic error in K_d from a single binding isotherm.[5]

Another pivotal area of research involves investigating method-dependent sources of systematic errors in determining K_d. Variations in methodologies for determining K_d result in differences in how the value of R is ascertained. Each method carries inherent potential for systematic errors in finding R.[6] It is imperative to delve deeper into understanding these sources, including their origins, their impact on the accuracy of R, and potential strategies for mitigation. One of the most glaring and persistent sources of systematic errors in R stems from the utilization of nonadditive signals. For example, while fluorescence anisotropy and light polarization are closely related, fluorescence anisotropy exhibits additivity, whereas light polarization does not. Nonetheless, polarization is not only commonly used instead of fluorescence anisotropy but is also integrated as the default output of some commercial

Published: May 22, 2024



instruments, e.g., in Synergy H4 (BioTek Instruments Inc.) and Infinite 200 Pro (Tecan). Therefore, addressing method-dependent sources of systematic errors in K_d determination is paramount for improving accuracy of K_d values across different methods.

Educational initiatives warrant greater attention from the research community than they currently receive. K_d determination is primarily practiced by chemical biologists, molecular biologists, medicinal chemists, and biochemists. Textbooks used for undergraduate studies in these disciplines often provide only superficial discussions on K_d determination, neglecting to address the sources, extents, and implications of inaccuracies in K_d. Additional materials detailing these aspects should be published as online supplements so that course instructors can guide students to such materials when seen necessary. Manuals of instruments used for K_d determination should also be augmented with this crucial information.

As part of educational efforts, it is crucial to equip authors, reviewers, and editors with a consensus set of requirements for the quality of binding experiments and presentation of their results. Similar recommendations have been developed, adopted, and implemented by scientific journals and publishers across various research fields,[7] indicating the feasibility of adopting analogous practices for K_d determination. The necessity to establish such requirements for K_d determination has previously been highlighted by Jarmoskaite et al.,[1] although that call did not seem to garner much traction. I echo this suggestion and, to kick-start this dialogue, propose the following requirements as a starting point.

1) Authors should articulate accuracy requirements for K_d, tailored to the intended applications of the scrutinized affinity complexes.
2) For K_d values necessitating high precision, employing multiple methodologies for determination should be standard practice.
3) Efforts should be made and reported to minimize systematic errors in concentrations and R, or, where feasible, eliminate them altogether.
4) Experimental validation should confirm the stability of K_d values against deliberate changes in the concentration of the limiting component and incubation time.
5) Presentation of K_d values should adhere to a judicious selection of the number of significant figures representing real precision of the determined K_d, which cannot be greater than that of the least precise of the three variables (two concentrations and R)
6) Complete transparency should be maintained by publishing not only details of experimental conditions but also raw data and tabulated binding isotherms.
7) As methodologies for assessing systematic errors in K_d emerge, their application should be integrated into the K_d-evaluation process.

Given the expansive community of researchers and practitioners engaged in K determination, a dedicated forum may prove invaluable for fostering productive discourse. The author created a "SC-Requirements for K_d determination" Microsoft Team for this purpose.[8] Please, send requests for access to this forum by e-mail to skrylov@yorku.ca; your e-mail address will then be added to this Team.

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https://pubs.acs.org/10.1021/acsmeasuresciau.4c00023

Notes

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(7) A curated, informative and educational resource on data and metadata standards, inter-related to databases and data policies. https://fairsharing.org.
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https://doi.org/10.1021/acsmeasuresciau.4c00023
ACS Meas. Sci. Au 2024, 4, 231−232