Simultaneous Determination of K_d and Protein Concentration

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Abstract

Non-equilibrium capillary electrophoresis of equilibrium mixtures (NECEEM) is a versatile tool for studying affinity binding. Here we describe a NECEEM-based approach for simultaneous determination of both the equilibrium constant, K_d, and the unknown concentration of a binder. We have fully validated this approach *in silico*, and applied it to an experimental model. The general approach described here is applicable not only to NECEEM, but to other methods that can determine the fraction of unbound molecules at equilibrium.

Introduction

Biomolecular affinity interactions play a central role in cellular regulation and in biomedical research. An important parameter that describes such interactions is the equilibrium dissociation constant (K_d). For a binding reaction between a target (T) and a ligand (L), with the formation of an affinity complex (TL), K_d is expressed by the equation:

$$K_d = \frac{[T]_{eq}[L]_{eq}}{[TL]_{eq}}$$

where $[T]_{eq}$, $[L]_{eq}$, and $[TL]_{eq}$ are concentrations of the components at equilibrium.

NECEEM is an affinity method which relies on separation of T, L, and TL by capillary electrophoresis. This separation allows the measurement of the fraction of unbound ligand, *R*, which in turn allows the calculation of either K_d or the concentration of T, while the other one is known. In some cases, however, both the K_d and the concentration of T are unknown – examples include difficult-to-purify or unstable proteins. We have developed an approach that allows us to find both unknowns simultaneously.

In the following examples, we assume that K_d and the stock concentration of the target, $[T]_{stock}$, are unknown. They are determined in two steps.

On-line version

The Approach

Step 1. The goal is to find the dilution factor, *a*, of $[T]_{stock}$ that results in an *R* value approximately equal to 0.5. Importantly, the initial concentration of the ligand, $[L]_0$, is kept constant at the lowest value that can be reliably quantified by the detection technique of choice.

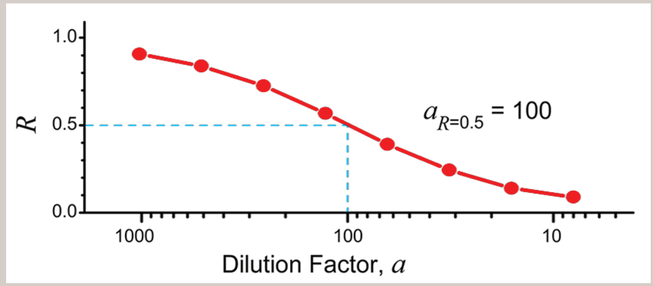

Figure 1. Example of dependence of fraction of unbound ligand (*R*) on dilution factor of target (*a*). In this case, dilution factor of 100, corresponding to *R*=0.5, will be used in step 2.

Step 2. Keeping the $[T]_0$ constant at the dilution from step 1, $[L]_0$ is varied until *R* approaches 0.9. The experimental results, presented as R versus concentration of $[L]_0$, are fitted with a theoretical dependence of these variables in which K_d and the concentration of T are used as fitting parameters. These parameters are determined as those resulting in the best fit.

$$R = -\frac{K_d + [T]_0 - [L]_0}{2[L]_0} + \sqrt{\left(\frac{K_d + [T]_0 - [L]_0}{2[L]_0}\right)^2 + \frac{K_d}{[L]_0}}$$

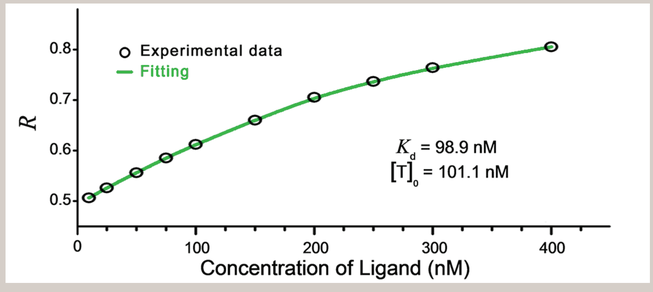

Figure 2. Example of fitting of dependence of fraction of unbound ligand (*R*) on concentration of ligand ($[L]_0$). Fitting yields both of the unknowns as parameters.

Lab Webpage

Validation

The approach was validated by *in silico* simulations of NECEEM across a range of initial conditions. The simulated electropherograms with pre-determined values of unknowns were processed as experimental data. The errors between the pre-determined and the resultant values are depicted below.

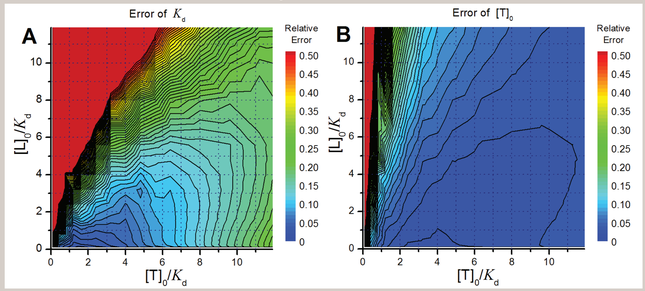

Figure 3. Relative error in determination of K_d (panel A) and $[T]_0$ (panel B) depending on ratio of $[L]_0$ and $[T]_0$ to K_d.

The results of the *in silico* study suggest how initial conditions of the experiment can be optimized to yield the most accurate results, and demonstrate the remarkable stability of the developed approach.

Application

The validated approach was used to determine the unknown concentration of MutS protein and K_d of its interactions with a DNA aptamer.

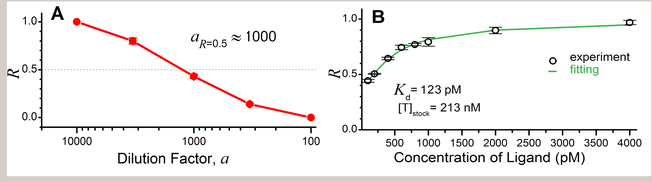

Figure 4. Application of the approach to an experimental model of MutS protein and its DNA aptamer. Steps 1 and 2 are depicted in panels A and B, respectively.

The results suggest that the previously-measured K_d was significantly overestimated by a single-point NECEEM approach. The determined concentration of the protein is in perfect agreement with measurement by BCA protein assay.

Reference: Kanoatov et al. *Anal. Chem.* 2015, 87, 3099-3106.