Tracer sedimentation equilibrium: a powerful tool for the quantitative characterization of macromolecular self- and hetero-associations in solution

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Abstract

During the last two decades the measurement and analysis of sedimentation equilibrium has been used increasingly as a method for characterizing intermolecular interactions in solution. More recently, a variant of this technique has been developed, in which a trace amount of a single solute component is labelled so that the concentration gradient of that component at sedimentation equilibrium may be measured independently of the gradients of all other components. The dependence of equilibrium tracer gradients upon solution composition may be readily interpreted in the context of models for self- and hetero-association of the labelled component, and, in the case of concentrated solutions, repulsive as well as attractive solute-solute interactions. We present a summary of experimental and analytic methods, a brief review of some previously published applications, and a preview of new applications demonstrating capabilities beyond those afforded by other current techniques for characterizing macromolecular associations in solution.

Quantitative analysis of macromolecular association equilibria via tracer methods: general considerations

In the present work we shall consider a solution containing macromolecular solute components A, B, C..., which may reversibly combine to form various species of stoichiometry $A_aB_bC_c\ldots$, where at least one of the stoichiometric coefficients $a, b, c\ldots$ is non-zero. Let us denote the molar concentration of each of these species as $c_{abc\ldots}$. Under thermodynamically ideal conditions, one may define the equilibrium constants

$$K_{abc\ldots} = \frac{c_{abc\ldots}}{c_{Aa}c_{Bb}c_{Cc}\ldots}$$

(1)

The objective of any quantitative study of reversible macromolecular associations at equilibrium is to determine how many species are present in detectable concentration under a given set of experimental conditions, and to evaluate all significantly non-zero values of the relevant equilibrium constants. In general this is done by experimental measurement of one or more composition-dependent equilibrium properties of the solution, followed by quantitative analysis of the composition dependence of the observed property or properties.

One particularly useful subset of the wide variety of techniques developed to study reversible associations consists of tracer methods, based upon detection of a solute component whose behaviour may be monitored independently of the concentration of other solute components present. The ability to detect a single component in a mixture of components may be due to the presence of a unique label, either natural or synthetic, or due to the use of a chemically specific detection scheme such as an immunoassay (in the case of a synthetic label, the labelled and unlabelled variants of the same molecule may be formally regarded as separate components). Let us define component A as the tracer component, and define a generic measurable property or signal $S_i$, the magnitude of which is linear in the concentrations of all species and in the amount of tracer component in each species. The contribution of each species i to the total signal is then

$$S_i = \varepsilon_i n_{A,i} c_i$$

(2)

where $n_{A,i}$ is the number of moles of A in the $i$th species and $\varepsilon_i$ is a signal-specific constant of proportionality characteristic of a particular solute species. (For example, if $S$ were absorbance, $\varepsilon_i$ would be a molar extinction coefficient.) It follows from eqn (2) that the total signal, normalized to the total concentration of tracer, is given by

$$S(c_{A,\text{tot}}, c_{B,\text{tot}}, c_{C,\text{tot}}\ldots) = \frac{\sum_i n_{A,i} c_i \varepsilon_i}{\sum_i n_{A,i} c_i}$$

(3)

where $c_{X,\text{tot}}$ denotes the total concentration of component X. Analysis of the observed dependence of $S$ upon the total concentrations of each component requires the formulation of models for association that permit the calculation of all $c_i$ as functions of the postulated $K_{abc\ldots}$ and the total critical information about the system.
concentrations of all solute components (see for example [1–3]).

### Tracer sedimentation equilibrium in an ideal solution containing an arbitrary number of solute components

A general treatment of sedimentation equilibrium in a solution containing an arbitrary number of interacting solute components and species at arbitrary concentration has been recently presented [4]. A simplified analysis applicable to ideal (dilute) solutions was presented earlier [5]. The results of this analysis are summarized below.

Consider an ideal solution containing solute components A, B, C, ..., where component A is labelled so that its concentration (or relative concentration) may be measured as a function of position in the centrifuge cell, independent of the concentrations of all other components. At sedimentation equilibrium, the total w/v concentration of A at radial position \( r \) is given by

\[
    w_A(r) = \sum_i f_{A,i}w_i(r) = w_A(r_0) \exp \left( \frac{M_{w,A}^\omega \omega^2}{2RT} (r^2 - r_0^2) \right)
\]

where \( f_{A,i} \) denotes the mass fraction of component A in species i, \( w_i(r_0) \) the w/v concentration of species i at radial position \( r_0 \), an arbitrarily selected reference position, \( \omega \) the angular velocity of the rotor, R the molar gas constant and \( T \) the absolute temperature. \( M_{w,A}^\omega \) denotes the weight-average buoyant molar mass of component A, given by

\[
    M_{w,A}^\omega = \frac{\sum_i f_{A,i}w_iM_i^\omega}{\sum_i f_{A,i}w_i} = \frac{\sum_i n_{A,i}c_iM_i^\omega}{\sum_i n_{A,i}c_i}
\]

where \( M_i^\omega \) denotes the buoyant molar mass of species i.

Since \( M_{w,A}^\omega \) is a function of species concentrations that vary as a function of radial position across the centrifuge cell, it is in principle also a function of radial position. However, in a short solution column and/or at rotor speeds such that component concentrations vary by a factor of approximately four or less, it may be shown that the value of \( M_{w,A}^\omega \) varies only slightly across the cell, and that the cell-average value \( \langle M_{w,A}^\omega \rangle \) is a close approximation to the value of \( M_{w,A}^\omega \) characteristic of the solute composition of the solution loaded into the cell [2,5,6]. The value of \( \langle M_{w,A}^\omega \rangle \) may be obtained experimentally by non-linear least-squares fitting of eqn (1) with \( M_{w,A}^\omega \) to the experimentally obtained values of \( w_A \) as a function of \( r \), or by linear least-squares fitting of a straight line to the transformed dependence of ln \( w_A \) (or ln S) upon \( r^2 \), the slope of which is equal to \( \langle M_{w,A}^\omega \rangle \omega^2/2RT \). In this fashion, the primary data \( w_A(r) \), obtained from solutions of varying composition, are converted into a set of secondary data, namely \( M_{w,A}^\omega \) as a function of the loading concentrations of each of the solute components.

### Experimental

A typical tracer sedimentation equilibrium experiment to detect and characterize the association between two macromolecular solutes (A and B) would involve the preparation of a series of solutions containing a fixed small amount of tracer A* sufficient to give the appropriate signal level, together with different amounts of unlabelled B. Each of these solutions is centrifuged to sedimentation equilibrium, and the radial gradient of tracer signal measured as described below. Any effect of unlabelled B upon the signal gradient of A* will reflect interactions between molecules of A* and B (Figure 1).

The gradient of tracer concentration at sedimentation equilibrium is experimentally characterized by measuring the
Intermolecular Associations in 2D and 3D

Figure 2 | Experimentally measured gradients of tracer

(A) Equilibrium gradients of a chromophorically labelled fibrinogen-binding peptide in the absence (○) and presence (●) of unlabelled fibrinogen, and unlabelled fibrinogen with a competing unlabelled peptide (×). (B) Equilibrium gradients of radiolabelled complement subcomponent C1r in the absence (●) and presence (●, ○) of increasing concentrations of unlabelled subcomponent C1s (the open circles indicate the highest concentration of unlabelled subcomponent C1s).

<table>
<thead>
<tr>
<th>Tracer</th>
<th>Description</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Chromophorically labelled fibrinogen-binding peptide</td>
<td>Unlabelled fibrinogen</td>
</tr>
<tr>
<td>B</td>
<td>Radiolabelled complement subcomponent C1r</td>
<td>Unlabelled subcomponent C1s</td>
</tr>
</tbody>
</table>

Examples of equilibrium gradients of a chromophorically labelled tracer are shown in Figure 2(A). In a post-centrifugation measurement, following centrifugation to sedimentation equilibrium in a preparative ultracentrifuge, a precision microfractionator is used to divide the contents of a centrifuge tube into fractions corresponding to laminae of radial thickness as small as 0.1 mm. The concentration of tracer in each of these radial fractions may then be determined by any of a variety of methods, including measurement of enzyme activity [8], specific colorimetric assays [4,9], specific binding assays [10–12] and measurement of the activity of radiolabel [2,13], in order to reconstruct the equilibrium gradient of tracer [7]. Examples of equilibrium gradients of a radiolabelled tracer are presented in Figure 2(B).

Successful application of tracer sedimentation equilibrium methodology depends very much upon the use of a label that provides the necessary sensitivity and linearity, but without perturbing the interactions to be studied. Extrinsic chromophoric or fluorophoric labelling is a very common way of creating a tracer protein or nucleic acid [14]. Although traditional protocols tend to insert the label at multiple residues, the use of recombinant technology has enhanced the ability to perform site-specific labelling [15,16]. When chromophoric labels are used, it is necessary to establish that (1) the physicochemical behaviour of the labelled macromolecule is not significantly perturbed by the introduction of label and that (2) the magnitude of signal for a fixed concentration of label is insensitive to variation in the concentration of unlabelled species present (see for example [17]).

References to published studies of macromolecular interactions in solution carried out using tracer sedimentation equilibrium are tabulated in Table 1.

Advantages of tracer sedimentation equilibrium

Increased resolution

It is evident upon comparison of eqns (3) and (5) that $M^*_A$ is an instance of the normalized generic signal $S_{A,tot}/w_A$, for the case that $\varepsilon_i = M^*_i$. A major advantage that tracer sedimentation equilibrium enjoys over other methods of studying macromolecular association equilibria in solution lies precisely in the nature of the property being measured – buoyant mass. In contrast to the situation for an arbitrarily selected spectroscopic or hydrodynamic signal, the value of each $\varepsilon_i (= M^*_i)$ may be calculated a priori due to the additivity of component contributions. For species $A, B, C, \ldots$

$$M^*_{ABC} = a M^*_A + b M^*_B + c M^*_C + \ldots$$

where $M^*_A, M^*_B, \ldots$ denote the independently measurable buoyant molar masses of pure (monomeric) A, B, C, respectively [5,6]. Hence all of the $\varepsilon_i$ within any given association scheme may be fixed a priori, and not treated as unknown, freely variable parameters. This prior knowledge of the values of all $\varepsilon_i$ greatly reduces the ambiguity that is otherwise encountered when fitting an association scheme to the experimentally measured dependence of $S_{A,tot}/w_A$ upon solute composition. Concrete examples of this advantage will be presented in a future article.
Table 1 | Experimental studies of macromolecular association equilibria via tracer sedimentation equilibrium

<table>
<thead>
<tr>
<th>System studied</th>
<th>Tracer signal</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hetero-association of apolipoproteins A-2 and C-1</td>
<td>Chromophoric label</td>
<td>[23]</td>
</tr>
<tr>
<td>Self-association of ovalbumin at high concentration</td>
<td>Chromophoric label</td>
<td>[17]</td>
</tr>
<tr>
<td>Self-association of aldolase at high concentration</td>
<td>Chromophoric label</td>
<td>[17]</td>
</tr>
<tr>
<td>Interaction of actin with actobindin</td>
<td>Chromophoric label</td>
<td>[24]</td>
</tr>
<tr>
<td>Hetero-association of aldolase and actin</td>
<td>Chromophoric label</td>
<td>[25]</td>
</tr>
<tr>
<td>Hetero-association of BSA and actin</td>
<td>Chromophoric label</td>
<td>[25]</td>
</tr>
<tr>
<td>Ca²⁺-linked self-association of complement subcomponent C1s</td>
<td>Radiolabel</td>
<td>[1]</td>
</tr>
<tr>
<td>Coupled self-association of lambda CI repressor and hetero-association with DNA</td>
<td>Chromophoric label</td>
<td>[26]</td>
</tr>
<tr>
<td>Binding of a platelet glycoprotein IIb peptide to fibrinogen</td>
<td>Chromophoric label</td>
<td>[27]</td>
</tr>
<tr>
<td>Ca²⁺-linked hetero-association of complement subcomponents C1r and C1s</td>
<td>Radiolabel</td>
<td>[2]</td>
</tr>
<tr>
<td>Hetero-association of fibrinogen with platelet fibrinogen receptor</td>
<td>Radiolabel</td>
<td>[13]</td>
</tr>
<tr>
<td>Self-association of fibrinogen in the presence of high concentrations of BSA</td>
<td>Radiolabel, chromophoric label</td>
<td>[9]</td>
</tr>
<tr>
<td>Self-association of tubulin in the presence of high concentrations of dextran</td>
<td>Radiolabel</td>
<td>[9]</td>
</tr>
<tr>
<td>Self-association of FtsZ in the presence of high concentrations of BSA or haemoglobin</td>
<td>Radiolabel</td>
<td>[28]</td>
</tr>
</tbody>
</table>

Feasibility of application to study of complex systems

Both conventional and tracer sedimentation equilibrium have been used successfully to characterize macromolecular self-associations in solutions containing one solute component [1,18–21], and hetero-associations in solutions containing two solute components (see [5,22] and references therein). Tracer sedimentation equilibrium has also been used successfully to characterize associations in a solution of two solute components in which macromolecular self- and hetero-association were present simultaneously [2]. We here demonstrate the feasibility of using tracer sedimentation equilibrium to characterize ternary interactions in solutions containing three solute components.

Consider a system containing tracer component A and two unlabelled components B and C. We postulate the existence of equilibrium complexes of the form ABₙCₘ, with

\[ K_{nm} = \frac{c_{AB}c_c}{c_{A}c_{B}c_{C}^{m+n}} \]  

(7)

and \( K_{00} = 1 \) by definition. We further postulate that it is possible to detect A at concentrations substantially smaller than the smallest experimentally employed concentrations of B and C, so that over the entire range of compositions for which data are obtained, \( c_B \approx c_{B,tot} \) and \( c_C \approx c_{C,tot} \). Under such conditions, the fraction of A present as a particular species is given by

\[ f_{A,nM} = \frac{c_{AB}c_c}{c_{A,tot}c_{B,tot}c_{C,tot}} \]

(8)

and the weight-average buoyant molar mass of A is given by

\[ M_{w,A} = \sum_{n,m} f_{A,nM}(M_A + nM_B + mM_C) \]

(9)

If all complexes of tracer A are monomeric in A as postulated here, then eqns (8) and (9) indicate that \( M_{w,A} \) will be independent of the concentration of A. In Figure 3, \( M_{w,A} \) is plotted as a function of log \( c_{B,tot} \) and log \( c_{C,tot} \) for three different association schemes, two of which involve the formation of different ternary complexes (more complex

Figure 3 | Dependence of \( M_{w,A} \) upon log \( c_{A,tot} \) and log \( c_{B,tot} \), calculated using eqns (8) and (9) with \( M_A^* = 1, M_B^* = 1.5, M_C^* = 2 \), and association constants of \( K_{00} = 1 \) and all other \( K_{nm} = 0 \) unless specified otherwise.

Left-hand panel: \( K_{10} = K_{01} = 1 \) (two binary complexes). Middle panel: \( K_{10} = K_{01} = K_{11} = 1 \) (two binary complexes + one ternary complex). Right-hand panel: \( K_{10} = K_{01} = K_{11} = 1 \) (two binary complexes + one ternary complex).
ternary association schemes and demonstrations of the ability of tracer sedimentation equilibrium analysis to discriminate between these schemes will be presented in a future article). It is evident that each scheme results in a functional dependence that may be clearly distinguished from those characterizing the other schemes, providing that experimentally measured values of $M_{w,A}^*$ are obtained with reasonable precision over a sufficiently large range of solution compositions, spanning 3–4 orders of magnitude in $c_{A,\text{tot}}$ and $c_{B,\text{tot}}$. Such measurements are well within the capability of tracer sedimentation equilibrium (see [1,2,13] for examples). If a proper tracer label has been chosen, wide variation in the concentrations of unlabelled reactants will result only in alteration of the dependence of tracer signal amplitude on radial position, and will not significantly alter the total amplitude of that signal integrated across the entire sample column.

In conclusion, tracer sedimentation equilibrium provides a uniquely powerful method for the detection and quantitation of macromolecular self- and hetero-associations over an unprecedentedly broad range of concentration, facilitating the study of both weak and strong intermolecular interactions in solution.

References


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