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Measurement of the Second Osmotic Virial Coefficient for Protein Solutions Exhibiting Monomer-Dimer Equilibrium

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Abstract

The second osmotic virial coefficient (B) is a measure of solution nonideality that is useful for predicting conditions favorable for protein crystallization and for inhibiting aggregation. Static light scattering (SLS) is the most common technique to determine B values, typically using protein concentrations less than 5 mg/mL. When performing SLS experiments at low protein concentrations, frequently the protein is either assumed to exist as a single non-associating species or a combination of assembly states independent of protein concentration. This manuscript examines the limit for ignoring weak reversible dimerization ($K_d \geq 1$ mM) by comparing B values calculated with and without accounting for self-association. Light scattering effects for equilibrium dimer systems with $K_d < 20$ mM and $K_d < 1$ mM will significantly affect apparent B values measured for 20 kDa and 150 kDa proteins, respectively. To interpret correctly light scattering data for monomer-dimer equilibrium systems, we use an expanded coefficient model to account for separate monomer-monomer (B_{22}), monomer-dimer (B_{23}), and dimer-dimer (B_{33}) interactions.

INTRODUCTION

The second osmotic virial coefficient (B) is a measure of non-ideal solution behavior resulting from two-body interactions. It is often denoted B_{22} to describe solute-solute interactions (for a two component system, the solvent is denoted by subscript 1 and the solute by subscript 2). Positive B_{22} values indicate net repulsive forces between solute molecules, while negative values show that net attractive forces between solute molecules present ¹. The most common methods used to determine second osmotic virial coefficients are static light scattering (SLS; also referred to in literature as laser light scattering or LLS) and sedimentation equilibrium. As has been pointed out by Winzor, when measured by light scattering, B_{22} values reflect not only protein-protein interactions but also contributions of protein-cosolute interactions to protein nonideality, whereas B_{22} values measured by sedimentation equilibrium provide an unequivocal measure of the osmotic virial coefficient for protein self-interaction ^{2,3}. However, as a measure of colloidal stability, B_{22} measured by light scattering provides a sound statistical-

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mechanical explanation that correlates with solution conditions conducive to protein crystallization^{1,3} and protein aggregation⁴.

Recent interest in methods for faster determination of B_{22} has produced novel approaches to SLS. Attri and Minton have developed a concentration gradient static light scattering method using continuous measurement of scattering and UV absorbance^{5,6}. Also, Bajaj et al. have proposed using a dual-detector cell for simultaneous measurement of scattered light intensity and protein concentration upon elution from SEC-HPLC to avoid peak broadening from interdetector delay volume in the standard online arrangement of these detectors in series⁷.

When used to model light scattering data for polydisperse self-associating systems according to the standard Debye plot equation (1), the B_{22} value represents net interactions and excluded volume effects of all solute species. The Debye plot equation is defined as follows:

$$\frac{Kc}{R_\theta} = \frac{1}{M} + 2B_{22} \quad (1)$$

In Eq. 1, R_θ is the excess Rayleigh ratio, c is the solute concentration, M is the solute molecular weight, and K is a constant defined by equation 2.

$$K = \frac{4\pi^2 n_0^2 (dn/dc)^2}{N_A \lambda_0^4} \quad (2)$$

In Eq. 2, N_A is Avagadro's number, n_0 is the refractive index of the solvent and λ_0 is the wavelength of incident laser light.

Commonly, protein solutions analyzed by SLS do not exceed a protein concentration of 5 mg/mL. However, the technique has been applied at higher protein concentrations to determine molecular weights and assembly states. For example, it has proved useful to observe self-association for insulin⁸ and bovine β -lactoglobulin (β -Lg)^{9,10}, the latter at concentrations up to 56 mg/mL. Timasheff et al. measured the reversible and rapid dimerization of bovine β -lactoglobulin (β -Lg) using sedimentation equilibrium (up to 70 mg/mL protein concentration) and static light scattering (up to 56 mg/mL protein concentration), with acceptable agreement between the two methods^{9,10}.

B_{22} values are of interest for solutions that contain both monomeric and higher order assembly states of proteins. As previously stated, when using Eq. 1 to model light scattering data for polydisperse self-associating systems, the B_{22} value represents net interactions and excluded volume effects of all species. For example, in a solution that contains both monomeric and dimeric protein species, we may wish to determine monomer-monomer, monomer-dimer, and dimer-dimer interactions. To determine separate coefficients for these interactions, we use an expanded coefficient model described in the following section. Furthermore, we show how apparent values of the coefficients measured by light scattering are affected by the presence of monomer-dimer equilibrium.

THEORY

The Debye plot equation (rewritten as Eq. 4) is derived from the virial expansion of osmotic pressure (Eq. 3)¹¹ as follows:

$$\frac{\Pi}{RT} = \frac{c}{M_w} + Bc^2 \quad (3)$$

$$\frac{Kc}{R_\theta} = \frac{-(\partial\mu_1/\partial c)}{RT\bar{V}_1} = \frac{(\partial\Pi/\partial c)}{RT} = \frac{1}{M_w} + 2Bc \quad (4)$$

where c is the total protein concentration (g/mL), M_w is the solute (protein) molecular weight, R_θ is the excess Rayleigh ratio, B is the second osmotic virial coefficient (mol·mL/g²), and $\bar{V}_1$ is the solvent partial molar volume. The Rayleigh equation, from which Eq. 4 originates, assumes ideal gas behavior when accounting for the number of independent light scattering centers per unit volume¹¹. In liquid solutions, scattering is caused by fluctuations in solvent and solute densities due to molecular motion. To extend the theory for Rayleigh scattering to liquid solutions, it is necessary to relate fluctuations in the solute concentration to fluctuations in polarizability by substituting the osmotic pressure gradient, $(\partial\Pi/\partial c)$, for the solvent chemical potential gradient $(\partial\mu_1/\partial c)$, as shown in Eq. 4. The validity of this substitution is straightforward to confirm. The time-average scattering intensity (expressed in terms of the scattering wave vector q), a function of the scattering angle and wavelength, is related to the product of the static structure and form factors $S(q)$ and $P(q)$ respectively). For Rayleigh scatterers, $P(q)$, an orientation-averaged function of particle size and shape, is essentially unity¹². For dilute solutions as $q \rightarrow 0$, $S(q)$, the Fourier transform of the radial distribution function (an averaged distribution of intermolecular distances), approaches $S(0) = RT/M(\partial\Pi_{\text{osm}}/\partial c)_{0,T}$ and the substitution in Eq. 4 is valid¹³.

For a system exhibiting monomer-dimer equilibrium, it is necessary to modify Eq. 3 as follows:

$$\frac{\Pi}{RT} = \frac{c_2}{M_2} + \frac{c_3}{M_3} + Bc^2 \quad (5)$$

In Eq. 5, B represents the total nonideality of the system, and the monomer and dimer quantities are denoted by subscripts 2 and 3, respectively. B may be expanded further to account for separate monomer-monomer (B_{22}), monomer-dimer (B_{23}), and dimer-dimer (B_{33}) interactions according to the monomer or dimer mol fraction (y_i) as follows:

$$B = y_2^2 B_{22} + y_3^2 B_{33} + 2y_2 y_3 B_{23} \quad (6)$$

Using the expansion of B shown in equation 6, equation 5 becomes:

$$\frac{\Pi}{RT} = \frac{c_2}{M_2} + \frac{c_3}{M_3} + B_{22}c_2^2 + B_{33}c_3^2 + 2B_{23}c_2c_3 \quad (7)$$

The monomer and dimer protein concentrations can be expressed as a function of the total concentration c and the dimer dissociation constant K_d ($= [M]^2/[D]$) while converting K_d from molarity to g/mL as follows:

$$c_2 = \frac{M_2}{4000} \left(\left(K_d^2 + 8000 \frac{K_d c}{M_2} \right)^{1/2} - K_d \right) \quad (8)$$

$$c_3 = \frac{M_3}{16000 K_d} \left(\left(K_d^2 + 8000 \frac{K_d c}{M_3} \right)^{1/2} - K_d \right)^2 \quad (9)$$

Substituting Eq. 5, Eq. 8 and Eq. 9 into Eq. 4, the SLS equation for a system exhibiting monomer-dimer equilibrium becomes:

$$\frac{Kc}{R_\theta} = \frac{K_d}{2M_2(K_d^2 + 8000K_d c/M_2)^{1/2}} + \frac{1}{2M_2} + 2Bc = \frac{1}{M_w} + 2Bc \quad (10)$$

Here, we have assumed that K is the same for all species, an assumption equivalent to assuming that the refractive index increment dn/dc is the same for all species. The simplified form of Eq. 10 uses the weight average molecular weight M_w , calculated from the value of K_d as a function of c . Substitution of the osmotic pressure gradient from Eq. 7 into Eq. 4 gives the expanded

Rayleigh equation accounting for monomer-monomer (B_{22}), monomer-dimer (B_{23}), and dimer-dimer (B_{33}) interactions:

$$\frac{Kc}{R_\theta} = \frac{(\partial\Pi/\partial c)}{RT} = \frac{\left(\begin{aligned} &2000K_dM_2 + 2000M_2(K_d^2 + 8000K_dc/M_2)^{1/2} \\ &+ 2B_{22}M_2^3K_d(K_d^2 + 8000K_dc/M_2)^{1/2} - 2B_{22}M_2^3K_d^2 \\ &+ B_{33}M_3^2K_dM_2(K_d^2 + 8000K_dc/M_2)^{1/2} \\ &+ 2000B_{33}M_3^2(K_d^2 + 8000K_dc/M_2)^{1/2} \\ &- B_{33}M_3^2K_d^2M_2 - 6000B_{33}M_3^2K_dc + 3B_{23}M_3K_d^2M_2^2 \\ &+ 12000B_{23}M_3K_dM_2c - 3B_{23}M_3K_dM_2^2(K_d^2 + 8000K_dc/M_2)^{1/2} \end{aligned} \right)}{4000M_2^2(K_d^2 + 8000K_dc/M_2)^{1/2}} \quad (11)$$

Note that the preceding section implies treats water and cosolutes such as buffers and salts as a single pseudosolvent, and thus these B_{ij} values incorporate contributions of cosolutes to the nonideality of the protein. Thus, these B_{ij} measured by light scattering are a useful measure of protein nonideality, but strictly speaking cannot be identified as osmotic second virial coefficients^{2,3}.

METHODS

Simulated light scattering data were generated using Eq. 10 using a range of K_d values and assuming ideal interactions ($B=0$). Experimental data for recombinant human interleukin-1 receptor antagonist (rhIL-1ra) and β -lactoglobulin were taken from previously published results^{14,15}.

RESULTS AND DISCUSSION

When performing SLS experiments at low protein concentrations (*i.e.* < 5 mg/mL), frequently protein molecules are assumed to exist either as a single non-associating species or as a combination of assembly states that are independent of protein concentration. However, simulated SLS data reveal that even very weak reversible dimerization (*i.e.* $K_d \geq 1$ mM) can significantly alter the observed scattering, causing the curvature characteristic of concentration-dependent self-association. The dramatic effect of increasing dimer concentration on simulated light scattering data is displayed in Figure 1, which would have a slope of 0 (for simulated B value of 0) across all protein concentrations in absence of monomer-dimer equilibrium. Figure 1 was generated using Eq. 10 for a range of dimer dissociation constants (K_d) from 1 to 20 mM and fixed B value of 0 for a 20 kDa protein, a molecular weight typical of many cytokines. Figure 1 shows that low dimer concentrations contribute significantly to reducing Kc/R and would lead to underestimation of B values if Eq. 4 were used under the (false) assumption that the average protein molecular weight is independent of protein concentration. The effect of the monomer-dimer equilibrium is even more apparent when the simulated data is plotted for protein concentrations up to 100 mg/mL (Figure 1B).

Figure 2 shows the same simulation in Figure 1, but for a protein molecular weight of 150 kDa, corresponding to that for a typical antibody. At a given total mass concentration of protein and K_d value, a much lower mol fraction of the protein is present as dimer than in Figure 1, and thus the assumption of a single scattering species is reasonable up to higher protein mass concentrations than for the 20 kDa protein (Figure 2A).

Table 1 summarizes the results of using Eq. 4 to fit the simulated data that is plotted in Figure 1 and Figure 2. The fit was performed only on data for a protein concentration range of 1 – 5 mg/mL. In addition to the deviation in B from the correct value of 0, the fit using Eq. 4 also

significantly underreports the presence of the dimer. For the 20 kDa protein at $K_d > 20$ mM, the effect on B from reversible dimerization is negligible and the assumptions for Eq. 4 are valid. For the 150 kDa protein at $K_d \geq 1$ mM, the effect on B from reversible dimerization is negligible and the assumptions for Eq. 4 are valid. Notably, the mass averaged molecular weight values fit using Eq. 4 for both the 20 kDa and 150 kDa protein are very close to the actual values for monomer. Thus, interpretation of light scattering data using Eq. 4 and a visual inspection of the data over a protein concentration range of 1 – 5 mg/mL, which appears linear as shown in Figure 1 and Figure 2, is unlikely to indicate the presence of a monomer-dimer equilibrium. Very similar results have been previously reported by Johnson, et al. in analysis of protein-protein interactions by ultracentrifugation¹⁶. They analyzed a case where monomer-dimer association was ideal, but the data was analyzed assuming that the solute was monomeric but non-ideal. As in the current work, they found that fits to the equivalent of Eq. 4 (erroneously) negative B values, even though the apparent molecular weights were nearly correct.

The challenge of interpretation of SLS data can be illustrated using recombinant human interleukin-1 receptor antagonist (rhIL-1ra), a 17.3 kDa cytokine inhibitor. The data presented is taken from previously published results¹⁴. Debye plots for static light scattering of rhIL-1ra at 25°C and protein concentrations of 1-6 mg/mL are shown in Figure 3. As shown in Table 2, when Eq. 10 is used to fit the data, in this concentration range rhIL-1ra appears to be monomeric ($M = 17.2$ kDa) over the solution ionic strength range of 0.025 – 0.184 molal, with protein-protein interactions becoming more attractive (increasingly negative B_{22} values) at lower ionic strength. However, the full static light scattering data collected from 1 to 100 mg/mL displayed in Figure 4 do not match the aforementioned explanation. Instead of a linear plot as in Figure 4 that would be expected if the rhIL-1ra samples were either entirely monomeric or contained a protein concentration-independent fraction of oligomers, the curvature at increasing protein concentration is characteristic of equilibrium self-association. The greater curvature at low ionic strength from smaller values of Kc/R is caused by the increased presence of higher weight average molecular weight species. Based on observations from dynamic light scattering, this higher molecular weight species is an equilibrium dimer¹⁴. To confirm the validity of using static light scattering at protein concentrations up to 100 mg/mL, osmotic pressure was measured directly using membrane osmometry at 25°C¹⁴. The dashed lines in Figure 4 show the fit using Eq. 10.

Estimates for the K_d for monomer-dimer equilibrium of rhIL-1ra and the overall B value as a function of ionic strength (determined by fitting Eq. 10 to the data of Figure 4) are displayed in Figure 5. It should be noted that K_d and B values can be determined simultaneously only if the data spans concentration ranges that are near to K_d , where sufficient curvature in the scattering plots exists. Otherwise, difficulties in simultaneously determining B and K_d may lead to artifacts¹⁶. Modeling all protein-protein nonidealities with coefficient (B) in Figure 5 shows a change in solution nonideality from net attractive to net repulsive interactions as ionic strength increases. The K_d values estimated from Eq. 10 are close to K_d values measured using sedimentation equilibrium experiments¹⁴. Note the B values fit using Eq. 10 are an ‘average’ over the total protein concentration range and do not account for the changing excluded volume contributions due to monomer and dimer concentrations that change with total protein concentration. However, the overall scattering signal is dominated by the contribution of increasing dimer concentration compared to the contribution from solution nonideality represented by B , which allows Eq. 10 to be used to estimate K_d values.

Using values of K_d determined by sedimentation equilibrium¹⁴, the data in Figure 4 was refit for B_{22} , B_{33} and B_{23} values using Eq. 11 and the results are displayed in Figure 6. Values for B_{22} , B_{33} and B_{23} normalized to the hard sphere excluded volume are displayed in Table 3 and

show monomer-monomer and dimer-dimer interactions are repulsive (positive values), whereas monomer-dimer interactions are attractive (negative values).

The negative values for monomer-dimer interactions (B_{23}) deserve special comment.. If the charge per mass were constant during the dimerization of this protein, and further, if any charge effects can be modeled as uniformly distributed over the protein's surface, we should expect that Donnan effects on B's would be constant and the differences in B between monomer and oligomers at a given ionic strength would reflect only excluded volume contributions. Clearly, negative values for monomer-dimer interactions cannot be explained in terms of excluded volume effects alone. In other recent work ¹⁷ we have shown that aggregation of IL-1ra in solution proceeds through reaction of a dimer and a monomer to form a trimeric species, with overall aggregation reaction kinetics that are third-order in protein concentration. Thus, the observed negative values for B_{23} are consistent with the net attractive interactions that result in IL-1ra trimer formation. The difference in sign observed between B_{23} and B_{22} or B_{33} values for rhIL-1ra emphasizes the point that net protein-protein interactions are not determined by uniform bulk colloidal properties; instead, B values are governed by a few highly attractive or repulsive interactions resulting from configurations with high geometric complementarity ¹⁸. Additionally, nonidealities resulting from cosolute-protein interactions may contribute to the overall nonidealities detected by light scattering ^{2,3}.

CONCLUSIONS

When analyzing static light scattering data, even a weak monomer-dimer equilibrium (K_d values less than 20 mM for a 20 kDa protein) will noticeably affect the B_{22} values determined using the standard Debye plot equation over a protein concentration range of 1 – 5 mg/mL. For a 150 kDa protein, a monomer-dimer equilibrium with K_d values less than 1 mM will have a significant effect on the calculated B_{22} value calculated in similar fashion over a protein concentration range of 1 – 5 mg/mL. For monomer-dimer equilibrium K_d values less than these limits, the B_{22} value calculated using the standard Debye plot equation will have a significant negative contribution that is due to the concentration-dependent formation of equilibrium dimer. To correctly measure nonideality for a monomer-dimer equilibrium system, we have developed an expanded coefficient model to quantify separate monomer-monomer (B_{22}), monomer-dimer (B_{23}), and dimer-dimer (B_{33}) interactions.

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Figure 1.

Simulated data using Eq. 10 showing the effect of an equilibrium dimer on the light scattering signal in the ideal solutions ($B = 0$). Graphs A and B: 20 kDa protein for protein concentration ranges of 0–0.01 g/mL and 0–0.1 g/mL respectively. In both graphs $K_d = 20, 10, 5, 2$, and 1 mM from the upper to lower curve, respectively.

Figure 2.

Simulated data using Eq. 10 showing the effect of an equilibrium dimer on the light scattering signal in ideal solutions ($B = 0$). Graphs A and B: 150 kDa protein for protein concentration ranges of 0–0.01 g/mL and 0–0.1 g/mL respectively. In both graphs $K_d = 20, 10, 5, 2$, and 1 mM from the upper to lower curve, respectively.

Figure 3.

Static light scattering data for rhIL-1ra at 25°C and pH 6.5 over a protein concentration range of 1–6 mg/mL. Solid lines represent the fit using Eq. 10. Ionic strength: ○, 0.184 m; ◆, 0.111 m; ■, 0.091 m; ◇, 0.060 m; △, 0.031 m; ●, 0.025 m. Error bars are standard deviations from 200 measurements.

Figure 4. rhII-Ira static light scattering results at 25°C fit using Eq. 10. Ionic strength (m): ○, 0.184 m; ◆, 0.111 m; ■, 0.091 m; ◇, 0.060 m; △, 0.031 m; ●, 0.025 m. Error bars from the standard deviation are less than the symbol size.

Figure 5.

Graph A: K_d values. $\square$: light scattering (using Eq. 10), $\circ$: sedimentation equilibrium data of Alford et al.¹⁴. Error bars for sedimentation equilibrium represent 95% confidence intervals (upper confidence interval for sedimentation equilibrium at $I = 0.184$ m extends off the chart)

to 79.9 mM). Graph B: B values. □: light scattering (using Eq. 9). Error bars for sedimentation equilibrium represent 95% confidence intervals.

Figure 6.

rhIL-1ra static light scattering data at 25°C and pH 6.5 fit using Eq. 11 (dashed lines). Ionic strength (and respective buffer): ○, 0.184 m (46.5 mM citrate); ◆, 0.111 m (83.3 mM phosphate); ■, 0.091 m (20.8 mM citrate); ◇, 0.060 m (42.7 mM phosphate); △, 0.031 m (16.0 mM phosphate); ●, 0.025 m (4.5 mM citrate). Error bars from the standard deviation are smaller than the symbol size.

Table 1

Apparent B_{22} values obtained by using Eq. 4 to fit simulated data from Eq. 10. Simulated data were generated using a protein monomer molecular weight of 20,000 (top set of values) or 150,000 (lower set of values) and a B value of 0. The fit was performed only on data for a protein concentration range of 1 – 5 mg/mL. Note that apparent B_{22} values increasingly differ from the actual value of 0 as K_D decreases.

Kd(mM)	Actual Values Used in Equation 10		B (mol mL/g ²)	$B \times 10^4$ (mol mL/g ²)	Apparent Values Fit with Equation 4	
	Mol Fraction Dimer at 5 mg/mL protein				Mass Averaged Molecular Weight(Da)	Mol Fraction Dimer
1	0.27		0	-8.2E-04	21161	0.06
2	0.17		0	-6.4E-04	20474	0.02
5	0.08		0	-3.7E-04	20114	0.01
10	0.05		0	-2.1E-04	20034	0.002
20	0.02		0	-1.1E-04	20009	0.0005
1	0.06		0	-4.E-05	150426	0.003
2	0.03		0	-2.E-05	150120	0.0008
5	0.013		0	-8.E-06	150021	0.0001
10	0.007		0	-4.E-06	150005	0.00004
20	0.003		0	-2.E-06	150001	0.000009

Table 2

Apparent values using Eq. 10 to fit rhIL-1ra light scattering data in Figure 3. $B_{22}^{\text{HS}} = 1.66 \times 10^{-4} \text{ mol mL g}^{-2}$ was calculated as $B^{\text{HS}} = 2\pi N_A D^3 / 3M^2$ using a hard sphere radius D of 3.4 nm.

Ionic Strength (m)	$B_{22} \times 10^4$	$B_{22}/B_{22}^{\text{HS}}$	M_w
0.025	-5.0 ± 0.2	-3.0 ± 0.1	17.2 ± 0.1
0.031	-4.5 ± 0.2	-2.7 ± 0.1	17.2 ± 0.1
0.060	-4.2 ± 0.2	-2.5 ± 0.1	17.2 ± 0.1
0.091	-3.0 ± 0.2	-1.8 ± 0.1	17.2 ± 0.1
0.111	-2.6 ± 0.1	-1.6 ± 0.1	17.2 ± 0.1
0.184	-2.5 ± 0.2	-1.5 ± 0.1	17.2 ± 0.1

Table 3

rhIL-1ra dimer dissociation constants (37°C, pH 6.5 converted from values at 25°C) measured using sedimentation equilibrium¹⁴ and second osmotic virial coefficients (25°C, pH 6.5) measured using static light scattering. 95% confidence intervals were calculated using the respective 95% confidence intervals for the previously reported K_d values. The hard sphere excluded volumes were calculated using $B^{HS} = 2\pi N_A D^3/3M^2$, where N_A is Avagadro's number, M is the protein molecular weight (17267 Da for monomer from the amino acid sequence), and D is the hard sphere diameter. Hydrodynamic diameters measured using dynamic light scattering described elsewhere¹⁴ were 3.4 nm for monomer and 5.7 nm for dimer. A diameter of 4.6 nm and molecular weight of 25900 Da were used to calculate B^{HS} for monomer-dimer interactions.

	K_d (mM)	B_{22}/B^{HS}	Upper 95%	Lower 95%
0.025	3.8	2.8	4.6	1.1
0.031	4.2	2.2	3.9	0.5
0.060	5.4	1.7	3.4	0.1
0.091	7.5	0.9	2.5	-0.5
0.111	8.4	0.5	1.9	-0.9
0.184	12.6	0.1	1.5	-1.2
Ionic Strength (m)	K_d (mM)	B_{33}/B^{HS}	Upper 95%	Lower 95%
0.025	3.8	2.4	2.2	2.7
0.031	4.2	2.4	2.2	2.7
0.060	5.4	2.3	2.0	2.8
0.091	7.5	1.7	1.5	1.9
0.111	8.4	1.1	1.1	0.4
0.184	12.6	0.7	1.0	-19.3
Ionic Strength (m)	K_d (mM)	B_{23}/B^{HS}	Upper 95%	Lower 95%
0.025	3.8	-3.7	-3.2	-4.1
0.031	4.2	-3.5	-2.9	-4.0
0.060	5.4	-2.9	-2.2	-3.3
0.091	7.5	-1.4	-0.5	-1.8
0.111	8.4	-0.8	0.3	-1.2
0.184	12.6	0.1	4.3	-0.6