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ARTICLE *in* MOLECULAR PHARMACEUTICS · NOVEMBER 2014

Impact Factor: 4.38 · DOI: 10.1021/mp500533c · Source: PubMed

Article

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Mol. Pharmaceutics, **Just Accepted Manuscript** • DOI: 10.1021/mp500533c • Publication Date (Web): 12 Nov 2014

Specific Ion and Buffer Effects on Protein-Protein Interactions of a Monoclonal Antibody

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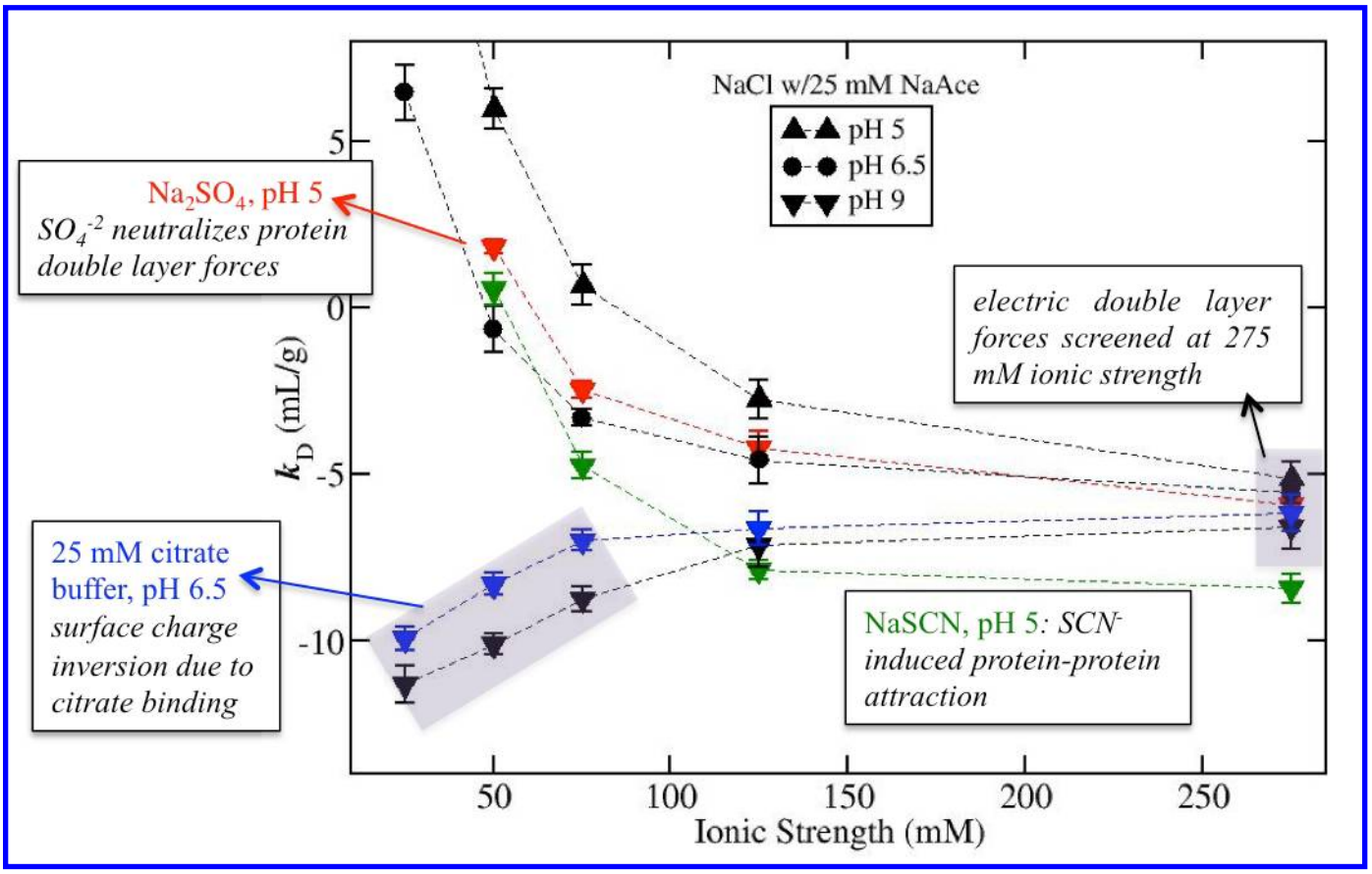
Abstract

Better predictive ability of salt and buffer effects on protein-protein interactions requires separating out contributions due to ionic screening, protein charge neutralization by ion binding, and salting-in(out) behaviour. We have carried out a systematic study by measuring protein-protein interactions for a monoclonal antibody over an ionic strength range of 25 to 525 mM at 4 pH values (5, 6.5, 8, 9) in solutions containing either sodium chloride, calcium chloride, sodium sulfate, or sodium thiocyante. The salt ions are chosen so as to represent a range of affinities for protein charged and non-charged groups. The results are compared to effects of various buffers including acetate, citrate, phosphate, histidine, succinate, or tris. In low ionic strength solutions, anion binding affinity is reflected by the ability to reduce protein-protein repulsion, which follows the order thiocyanate $>$ sulfate $>$ chloride. The sulfate specific effect is screened at the same ionic strength required to screen the pH dependence of protein-protein interactions indicating sulfate binding only neutralizaes protein charged groups. Thiocyanate specific effects occur over a larger ionic strength range reflecting adsorption to charged and non-charged regions of the protein. The latter leads to salting-in behaviour and, at low pH, a non-monotonic interaction profile with respect to sodium thiocyanate concentration. The effects of thiocyanate can not be rationalized in terms of only neutralizing double layer forces indicating the presence of an additional short-ranged protein-protein attraction at moderate ionic strength. Conversely, buffer specific effects can be explained through a charge neutralization mechanism, where buffers with greater valency are more effective at reducing double layer forces at low pH. Citrate binding at pH 6.5 leads to protein charge inversion and the formation of attractive electrostatic interactions. Throughout the report, we highlight similarities in the measured protein-protein interaction profiles with previous studies of globular proteins and of antibodies providing evidence that the behaviour will be common to other protein systems.

Keywords

Protein-protein interactions, specific ion effects, Hofmeister, monoclonal antibody, thiocyanate, second virial coefficients

Abstract Graphic



Introduction

There has been a renewed interest in determining specific ion and buffer effects in protein solutions at a fundamental level, but also because controlling solution behaviour is critical for many scientific and industrial applications. The original studies carried out more than a century ago by Hofmeister classified salts according to their effectivess at precipitating proteins.[1] Protein precipiation is still used as a separation process especially for industrial enzymes that are produced on large scales, but is also considered as an alternative separation strategy for therapeutic proteins. In addition, precipitating proteins to form crystals of diffraction quality depends sensitively on the nature of the salt and buffer type in the mother liquor. Some buffers such as dicarboxylic acids play a dual role as a buffer component and a cross linking agent for proteins.[2,3] Other salts are added to control protein solubility and alter the location of crystallizing mixtures on the phase diagram.[4] Of more interest recently, protein therapeutics are now being produced as liquid formulations often at high protein concentrations, where problems are encountered with phase separation, opalescence, high viscositives, and protein aggregation propensities. These processes can be controlled directly or indirectly by manipulating solution conditions to selectively alter protein-protein interactions. For instance, recent studies have linked antibody self interactions to protein aggregation,[5–7] liquid-liquid phase separation and opalescence,[8–14] and high viscosities.[15–23] While much has been learned about how ions interact with proteins, many questions still remain to be answered, especially concerning the link between protein-salt and protein-protein interactions as highlighted below.

Protein-Salt Interactions

In moderately concentrated salt solutions, there is a strong correlation between the preferential exclusion or adsorption of salt about a protein surface and the effect of salt on either protein stabilization or protein precipitation (salting-out). In the original work by Hofmeister, salts were classified according to their effect on the solubility of egg proteins.[1] The salting-out effectiveness of the ion decreases with the descending position of the ion in the Hofmesiter series, which is

given for anions by $SO_4^{-2} > HPO_4^- > OAc^- > Cl^- > Br^- > NO_3^- > I^- > SCN^-$ and for cations by $NH_4^+ > K^+ > Na^+ > Li^+ > Mg^{+2} > Ca^{+2}$. A strong correlation between the Hofmeister series for anions and their molal surface tension increment suggests salting-out is due to preferential exclusion of ions about the low dielectric interior of the protein,[24–32] which leads to an effective protein-protein attraction and protein precipitation.[33–35] A similar explanation has been invoked to explain the anion effectiveness for stabilizing proteins against unfolding, which correlates with the anion salting-out ability.[30,36–38] If exclusion is the only protein-salt interaction, then all salts would lower protein solubility due to having positive surface tension increments as also reflected by the universal salting-out of non-polar compounds.[24,39,40] Exceptions to this rule exist, the most notable being salts of divalent cations or chaotropic anions, both of which salt-in proteins at moderate salt concentrations[27,28,41,42] and lower protein melting temperatures.[38,43–47] The salting-in and protein destabilization behaviour is correlated with preferential interactions of salt to polar protein groups which have been quantified from the ability of salt to increase the aqueous solubility of model peptides,[48–52] to alter conformations of homopolypeptides and peptide-mimic polymers,[53–55] and from salt adsorption to peptide-mimic resins.[46,56] Thus the net effect of salt is determined by the competition between preferential adsorption to the protein surface and exclusion interactions due to the ion preference for hydration.[26–28] For anions, these effects are additive; increasing ion chaotropicity leads to increased adsorption, either by less preferential exclusion due to the surface tension effect or by increased binding interactions to the protein surface groups.[55,57–60] Conversely, divalent cations have high surface tension increments, but are salting-in agents and protein destabilizers due to preferential adsorption to polar surfaces.[26,28,50] As a consequence, the Hofmeister series for cations does not necessarily follow a ranking based on surface tension increments. The series for cations is not as pronounced as for anions, partly because the exclusion and adsorption effects balance each other.

The salting-in (out) interactions control behaviour at higher salt concentrations. For solutions at ionic strengths of 100 mM and below, the effect of salt type is manifested by differences in ion binding affinities to protein charged groups and the impact on electrostatic interactions.[35,61,62]

Specific ion binding effects have been characterized from the salt concentration required to refold proteins from an acid induced extended state to a molten globule.[63] Refolding in low ionic strength solutions at acidic pH is due to neutralizing the positive charge by ion binding. The anion binding affinity increases following the position in the descending Hofmeister series for monovalent anions. However, multivalent anions such as sulfate are high on the Hofmeister series, but also have high binding affinities, an effect which is attributed to stronger electrostatic interactions formed by multivalent over monovalent charges.[64,65] A similar series also occurs when ranking anions for their ability to bind positively charged resins providing support that anion binding sites are basic protein groups.[66] The strong binding of sulfate and monovalent chaotropic anions is also reflected by their ability to stabilize the helical state of synthetic polypeptides containing lysine or arginine at concentrations below 5 mM.[67–70] A similar specific ion dependence of binding to protein charged groups has also been observed from ζ-potential measurements,[71] NMR studies,[72] and isothermal titration calorimetry.[73] For simplicity, we use the nomenclature that ion binding corresponds to ion pair formation observed in low ionic strength solutions, which is reflected by electrostatic neutralization behaviour. Conversely salting-in interactions are used to describe weak preferential adsorption of salt that leads to an increase in protein solubility at moderate salt concentrations.

One of the key issues is identifying the sites for ion binding and the molecular forces involved. In low ionic strength solutions, the evidence is sufficiently strong that ion binding is occurring to protein charged groups. Ion binding affinities between dissolved anion and cations follow the so called law of matching water affinities "like likes like", that is pairs are the strongest for ions with similar water affinities, so that ion pairs between kosmotropes and between chaotropes are stronger than an ion pair formed between a chaotrope and a kosmotrope.[74] Because lysine and arginine are chaotropes, the law of matching water affinities provides a partial explanation why chaotropic anions bind more strongly to proteins. In addition, binding of chaotropic anions to lysine and arginine is enhanced due to preferential interactions with proximal non-polar groups.[58,60,75,76] The finding that ions adsorb to non-polar groups or surfaces is counter-intuitive, but is now well-supported from a range of experimental and simulation studies.[77–79] For monovalent cations, the affinity for

protein charged groups follows the law of matching water affinities as kosmotropic carboxylates have a preference for the more kosmotropic cation sodium over potassium.[80–83]. A study of specific cation effects on the lower cloud point temperature (LCPT) of an elastin-like polypeptide containing aspartic acid groups indicated calcium forms strong ion pairs with carboxylates.[84] The LCPT corresponds to the temperature above which a peptide or polymer undergoes a hydrophobic-induced collapse as reflected by solution clouding. In low ionic strength solutions, the main effect of increasing salt concentration is neutralization of the negatively charged groups by ion binding, which lowers the electrostatic barrier to aggregation. A langmuir type binding model described the data well consistent with the intuition that each charged group on a protein can only bind one ion. The dissociation constants K_d for divalent cations are on the order of 1 to 10 mM, which are at least ten times stronger than those of movovalent cations with values of between 78 mM and 345 mM.

The molecular details for salting-in effects observed in moderately concentrated salt solutions are still not well understood. Salting-in interactions have been characterized by the effect of salt concentration on the LCPT of polypeptides or peptide-mimic polymers such as poly-N-isopropylacrylamide (PNiPAM). In contrast to charged systems where ion binding lowers the cloud point due to an electrostatic neutralization effect, salt binding to uncharged peptide groups leads to salting-in or an increase in the LCPT, whereas a decreasing LCPT occurs due to salting-out effects. The salt-induced increase of the LCPT for two elastin-like polypeptide variants and PNiPAM provided evidence for chaotropic anion binding directly to the amide moiety of the peptide.[53,55] However, molecular simulations have found preferential adsorption of chaotropic anions occurs to the protein non-polar groups and not the amide moiety.[57,85–87] A recent study which is partially consistent with both views indicated that anions only adsorb to non-polar groups bound to electron withdrawing atoms, such as the α-carbon bound to the amide nitrogen.[59] In contrast, a detailed analysis of solubility data for uncharged peptides found that cations rather than anions form much stronger interactions with the peptide group,[50] a finding which has also been supported by recent molecular simulation studies.[57,88,89] The reason why salting-in effectiveness follows the reverse

Hofmeister series for anions was attributed to anion-cation association in solution, the strength of which is inversely correlated with the binding of the cation to protein groups. In addition, cation affinity for the peptide group can be enhanced by the binding of anions to nearby non-polar groups.[57,89] However, a spectroscopic study of the interaction between between butyramide and various cations found no evidence for an interaction of the amide group with either sodium or potassium and only a very weak interactions with divalent cations characterized by a dissociation constant of 8.5 M.[90] Along these lines, it has also been suggested that uncharged glycine peptides used for rationalizing cation specific effects[49] were not capped completely and contained negatively charged C-terminii groups.[91] If this were the case, the cation specific effects would be domninated by the interactions with the negatively charged groups[87] and lead to erroneous analysis for the binding interactions between cations and uncharged groups on the peptide backbone.

Linking to Protein-Protein Interactions

Understanding the link between protein-salt and protein-protein interactions requires measurements made as a function of salt concentration and salt type to separate out the competing effects from salting-in, salting-out and ion binding. Upper cloud point temperature (UCPT) measurements of protein solutions provide one such approach due to the ease of quantifying solution turbidity.[11,35,41,42] The UCPT corresponds to the onset of phase separation brought about by decreasing temperature for protein solutions. With increasing salt concentration, a decreasing cloud point indicates a salt-induced protein-protein repulsion (or salting-in), whereas an increasing cloud point reflects salt-induced attraction or salting-out. The relative contributions of protein-salt interactions to the UCPT were separated from each other in a study of lysozyme at pH 9.4.[35] Anion pairing neutralizes the positive protein charge reducing the electrical double-layer forces leading to an increase in the UCPT at low ionic strength. This effect could be captured using a langmuir binding term indicating one binding site per anion. The combined effects from salting-in and salting-out were accounted for by an empirical term that quantified the effect of salt addition on the energetics of the protein-salt/water interface. The position of the ion in the descending Hofmeister series was

correlated with the ability to neutralize the protein charge and the effectiveness at lowering the interfacial tension. Only chloride ion increased the interfacial tension, while all other anions investigated were chaotropic and lower the protein-solution interfacial tension leading to salting-in. The work confirmed that lysozyme solublity follows the reverse Hofmeister series in low ionic strength solutions due to binding chaotropic anions.[41,42,92–94] In more concentrated salt solutions, the competing effects of salting-in and salting-out cause lysozyme solubility to revert back to following the direct Hofmeister series.

A key issue to be resolved is whether or not the effect of anion binding in low ionic strength solutions can be rationalized in terms of changes to protein electrostatic properties. In the study of lysozyme solutions at pH 9.4, the UCPT corresponding to a neutralized protein is greatest in solutions of chaotropic anions.[35] The implication is that proteins complexed with chaotropic anions form more attractive protein-protein interactions. A similar deduction can be made when considering the UCPT for a monoclonal antibody measured at different pH values in solutions containing the potassium salts of either chloride, fluoride or thiocyanate.[11] The UCPT increases with decreasing pH in solutions containing potassium thiocyanate indicating protein-protein interactions become more attractive with more bound thiocyanate anions. Furthermore, the UCPT at pH 5.3 with a potassium thiocyanate concentration equal to 100 mM is greater than the UCPT for any of the salt solutions investigated at the protein isoelectric point. The finding that the thiocyanate-induced attraction is greater than the isoelectic attraction appears to indicate the effect of thiocyanate can not only be rationalized based on ion binding and protein charge neutralization.

While the UCPT measurements provide a rapid way for screening effects of solution conditions on protein behaviour, they reflect protein-protein interactions in concentrated protein solutions and at conditions removed from room temperature. The osmotic second virial coefficient provides a direct and rigorous measure of protein-protein interactions defined as the protein pair potential of mean force averaged over protein pair separation and relative orientations. For solutions of lysozyme, the value of B_{22} at the critical point depends on whether sodium chloride or ammonium sulfate is used as the precipitant indicating that there is no universal correlation between

dilute protein-protein interactions and the location of the cloud point line.[95] Furthermore, different forms of protein-protein interactions exhibit different temperature dependencies, that is, salting-out or hydrophobic-driven interactions exhibit a retrograde behaviour becoming more attractive with increasing temperature,[96] whereas the opposite temperature dependence is generally observed in low ionic strength solutions.[92] Thus extrapolating UCPT measurements to reflect room temperature protein interactions needs to be done with care. The additional advantage of characterizing protein-protein interactions from B_{22} values is that physical models can be used to match the data providing more detailed molecular insights.[97–99]

As yet, there have not been any exhaustive B_{22} studies examining specific ion effects as a function of pH and ionic strength, which is required to discriminate between the effects of ion binding, ion screening and salting-in interactions. Here, we provide one such study using a monoclonal antibody in solutions containing four different salts, sodium chloride, sodium thiocyanate, sodium sulfate, and calcium chloride. We have chosen to study sodium and chloride as models for neutral ions, while the other ions represent different extremes of the Hofmeister series. Sulfate, thiocyanate, and calcium all exhibit high relative binding affinities for protein charged groups at salt concentrations below 10 mM. However, sulfate is an effective salting-out agent at higher concentrations, while calcium and thiocyanate are effective at salting-in. While the values of B_{22} are measured at pH 5, for the other pH values, we characterize the protein-protein interactions in terms of a parameter k_D obtained from the slope of a plot of the protein diffusion coefficient versus protein concentration. Like B_{22}, the value of k_D is related to protein-protein interactions via integrals over the pair potential of mean force.[100–103] In the second part of the study, we examine the effects on protein-protein interactions of buffers including acetate, citrate, histidine, tris, or phosphate. Previous studies have already shown that antibody aggregation behaviour depends sensitively on the choice of buffer,[7,104] where the buffer specificity arises from effects on protein-protein interactions.[7,105–107] The experiments are carried out in two modes, one where the buffer concentration is fixed and k_D is measured as a function of ionic strength by adding sodium chloride. These results are contrasted with experiments where we vary the relative ratio of buffer and background

salt at fixed ionic strength. The latter experiments provide an indirect approach for quantifying the binding constant of the buffer to the protein.

Experimental

Sample preparation

mAb1 was stored as a liquid formulation at a protein concentration of 41 g/L with a 25 mM histidine buffer and 7 % w/v sucrose at pH 6.0. The protein has a sequence molecular weight equal to 144.8 kDa and the extinction coefficient of mAb1 is equal to 1.42 mL/(g-cm) at a wavelength of 280 nm.

Before each experiment, 4 litres of the buffer solution was prepared using a buffer concentration that fixed the ionic strength at either 5 mM, 10 mM or 25 mM as calculated according to the Henderson-Hasselbalch equation. All buffer solutions were filtered through a vacuum filtration unit using a 0.2 μ PES membrane (Stericup, Thermo Scientific Ltd., UK). The mAb1 formulation was concentrated to approximately 150 g/L using an Amicon Ultracentrifuge filter of 30K MWCO (Merck-Millipore Ltd., Ireland) and then placed in a Slide-A-Lyser dialysis cassette with a maximum volume of 3 mL and a 10K MWCO (Thermo Scientific Ltd., UK. The dialysis cassette was placed in 2 L of a dialysis buffer and stirred for 4 hours. The dialysis buffer was then exchanged and the dialysis was continued overnight. The dialyzed sample and the dialyzate were then saved and used for all subsequent sample dilutions.

Static light scattering

Static light scattering is used here for determining the osmotic second virial coefficient, B_{22}. For a system containing protein, water, and salt, the light scattering equation is given by [108–110]

$$\frac{Kc\,(\partial n/\partial c)^2_{T,\mu_w,\mu_s}}{\overline{R}_\theta} = \frac{1}{RT}\left(\frac{\partial \Pi}{\partial c}\right)_{T,\mu_w,\mu_s} = \frac{1}{M} + 2B_{22}c \qquad (1)$$

where subscripts w and s correspond to water and salt, respectively, $\overline{R}_\theta$ is the measured excess Rayleigh scattering ratio of the protein solution over the solvent, M is protein molecular weight, c is protein mass concentration, R is the gas constant, and Π is the osmotic pressure of the protein solution over that of the salt solution. K is the light scattering constant given by $2\pi^2 n_0^2/N_A\lambda^4$, where n_0 is the refractive index of the solvent, and λ is the wavelength of the light, and N_A is the Avogadro number. The second equality of Eq. (1) is true in the limit of low protein concentration such that higher order terms in the virial equation of state are negligible. $(\partial n/\partial c)_{T,\mu_w,\mu_s}$ is the refractive index increment of the protein solution measured at constant chemical potential of the water and salt μ_s and μ_w. In solutions at low salt concentration,

$$\left(\frac{\partial n}{\partial c}\right)_{T,\mu_w,\mu_s} \approx \left(\frac{\partial n}{\partial c}\right)_{T,p,m_s} \tag{2}$$

The refractive index increment at constant salt molality m_s has been measured extensively for protein solutions and is equal to approximately 0.185 L/g. In a light scattering experiment, $\overline{R}_\theta$ is measured for a series of samples with varying protein concentrations. A plot of the left side of Eq. (1) versus c yields a slope equal to $2B_{22}$ and the inverse of the y-intercept equal to M. The static light scattering experiments were carried out using a Wyatt miniDAWN TREOS detector connected to the Calypso which is an automated syringe delivery system with three pumps. Details of the experimental methodology and examples of typical experimental data and analysis are given in a previous publication.[111]

Dynamic light scattering

The intensity autocorrelation function $C(t)$ is measured in a dynamic light scattering experiment. The quantity of interest is the intermediate scattering function $g(q,t)$ which is given by[112]

$$C(t) = A + Bg(q,t)^2 \tag{3}$$

where A is an instrument-dependent optical constant and B is a background term, determined for each sample in the limit of large delay times. The intermediate scattering function is equal to the dynamic structure factor for the particles, which, in turn, is given by the density auto correlation function in space and time. For a monodispersed particle system, the density correlation function can be described by an exponential decay

$$g(q,t) = \exp(-Dq^2t) \tag{4}$$

where q is the scattering vector ($q = 4\pi n_0 \sin(\theta/2)/\lambda$); θ is the scattering angle), and D is termed the diffusion coefficient. Because protein solutions always contain a small amount of high molecular weight impurities, the scattering function needs to be fit to a population of decay times. In this case, relating the measured correlation function to the diffusion coefficient is an ill-conditioned problem because many different distributions can yield the same measured $g(q,t)$. A general approach to overcoming this problem for samples with uninodal distributions is to use the cumulant expansion. In this case, the natural logarithm of the correlation function is fit to a second order polynomial in the delay time

$$\ln g(q,t) = -q^2 D_z t + \frac{q^4 t^2}{2}(\delta D)_z^2, \tag{5}$$

where the subscript z denotes the z-average of the property. The first order coefficient in the expansion corresponds to the z-average of the diffusion coefficient. The second moment of the distribution is equal to the second order coefficient, which is the z-average in the fluctuations of the diffusion coefficient; larger fluctuations are related to broader size distributions. A measure of the polydispersity in the sample is often defined as $P = (\delta D)_z^2/D_z^2$, which is equal to zero for monodisperse samples.

The measured diffusion coefficient can be expanded in a series about protein concentration[100,101,103,113]

$$D = D_0\,[1 + k_D c] \tag{6}$$

where D_0 corresponds to the infinite dilution value of the diffusion coefficient, which is often reported in terms of the hydrodynamic radius R_h using the Stokes-Einstein relationship

$$R_h = \frac{k_B T}{6\pi\eta D_0} \tag{7}$$

The parameter k_D reflects the interactions between proteins, which controls their collective motion. Experimental studies have already shown a monotonic correlation between k_D and B_{22} indicating the parameter provides a direct measure of protein-protein interactions.[6,15,111,114]

Diffusion coefficients were measured using a DynaPro PlateReader (Wyatt, Santa Barbara, CA) at a laser wavelength of 838.88 nm with 30 microliter samples in a 384 well plate. Details of the experimental methodology, and sample data and analysis are given in a previous work.[111]

Electrophoretic mobility measurements

Electrophoretic mobility μ_E was measured by Massively-Parallel Phase Analysis Light Scattering (MP-PALS) using the Mobius manufactured by Wyatt Technology. μ_E corresponds to the velocity of charged particles under an applied electric field, which is quantified from the frequency (or Doppler) shift in the scattered light. The instrument contains a dynamic light scattering detector, which allows for simultaneous determination of electrophoretic mobility and particle diffusion coefficient. A 70 mW solid state laser at a wavelength of 532 nm provides light source and 30 detector channels are used to improve the sensitivity and speed of the measurement.

Each sample contained the protein at a concentration of 1 g/L obtained by diluting the protein sample after dialysis with the corresponding dialyzate. A syringe pump was used to inject 5 mL of the protein sample. An average ζ-potential was determined from a series of four measurements carried out on different sample volumes. The entire experiment was repeated for three sets of protein samples prepared from fresh dialysis runs. Error bars correspond to the standard deviation in the averages of the three independent measuremnts.

Results

pH and ionic strength dependence of protein-protein interactions

Specific ion effects manifested in solutions at low ionic strength are due to neutralization of protein charged groups by ion binding. In order to quantify these effects, we first consider how tuning the fixed charge on the protein (by changing pH) alters protein-protein interactions. In Figure 1 are shown measured values of B_{22} and k_D for mAb1 in sodium chloride solutions over a range of pH values. The results at pH values of 6.5 and below follow behaviour expected from considering how double layer forces change within Derajaguin-Landau-Verwey-Overbeek (DLVO) theory.[93,111,115–117] When electrical double layers overlap, there is an osmotic repulsion due to an increased concentration of ions in the overlapping double layers relative to the bulk solution. The excess ion density in the double layer is proportional to the net charge of the protein including any tightly bound ions. The decrease in B_{22} or k_D at fixed ionic strength reflects the reduction in double-layer forces due to lowering the net charge as the pH is increased (the isoelectric pH of mAb1 is approximately 9.1.[111]) The thickness of the double layer is determined by the inverse of the Debye-Huckel screening parameter κ, which scales with the square root of the ionic strength. When increasing ionic strength from 25 mM to 275 mM, the screening parameter κ^{-1} changes from approximately 2 nm to 0.6 nm. The impact of ionic screening on the protein-protein interactions is reflected by the corresponding decrease in B_{22} or k_D measured for solutions at pH values between 5 and 6.5.

A different pattern of protein-protein interactions occurs at pH 9. When approaching the pI of the protein, double layer forces are sufficiently weak due to lowering protein net charge. Under these conditions, attractive electrostatic interactions are formed between interacting surfaces with complimentary charge distributions.[111,118,119] The measurements for the mAb1 at pH 9 indicate attractive electrostatic interactions as the values of B_{22} and k_D both increase reflecting a reduction in protein-protein attraction over the ionic strength range where there is a large reduction in screening length. The key finding to emphasize is that, at an ionic strength of 275 mM, B_{22} or k_D values

are independent of the protein charged state providing strong evidence that electrostatic interactions are sufficiently screened. Thus, we use 275 mM as a rough estimate for delineating between electrostatic and non-electrostatic effects. Salt-induced changes at higher ionic strength are most likely related to salting-in or salting-out interactions or other forms of salt-induced repulsion or attraction.

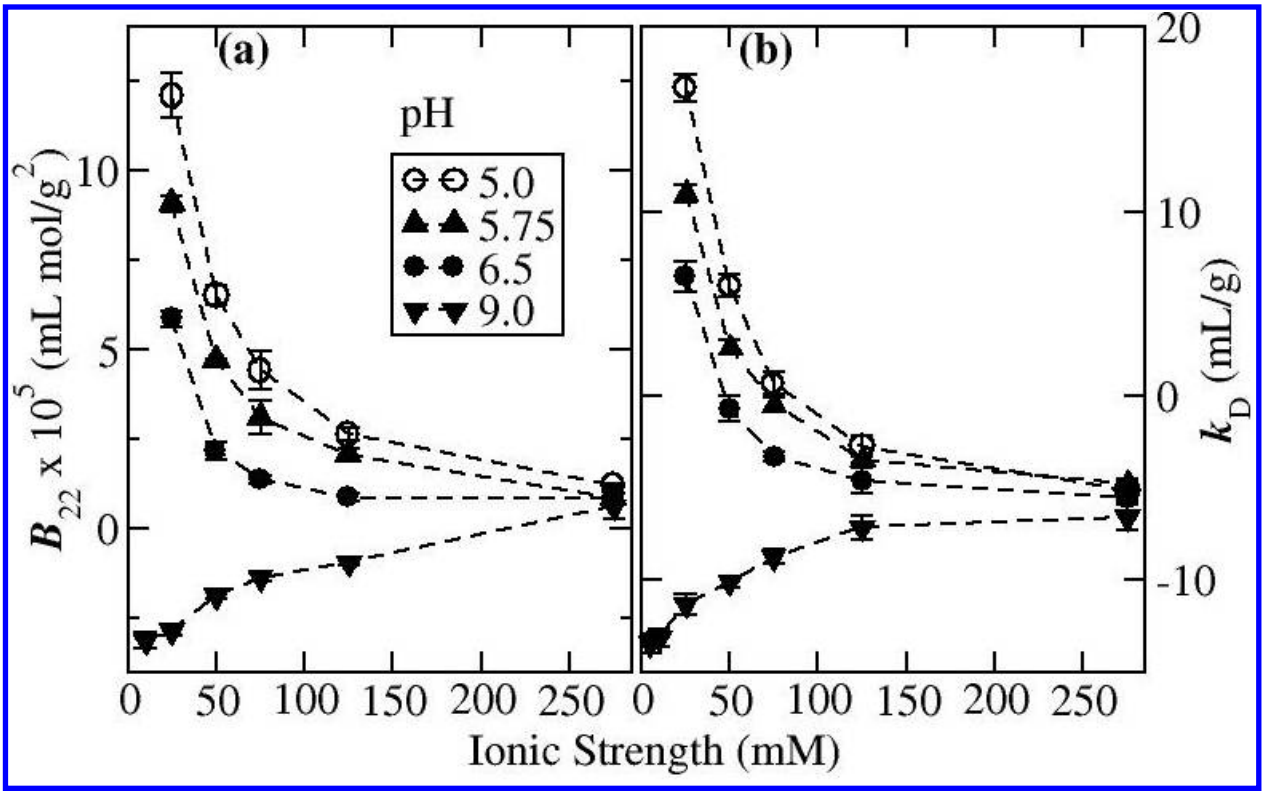


Figure 1: Protein-protein interactions measured for mAb1 in solutions of sodium chloride for different pH values in terms of **(a)** B_{22} or **(b)** k_D. For pH values between 5 and 6.5, a sodium acetate buffer is used at an ionic strength contribution of 25 mM. pH 9 corresponds to a tris buffer at an ionic strength contribution of 5 mM.

Measurements of protein-protein interactions in terms of k_D

In this work, we are characterizing protein-protein interactions in terms of either B_{22} or the interaction parameter k_D. B_{22}, according to McMillan-Mayer solution theory, has an exact statistical mechanical definition in terms of the potential of mean force.[120] However, in linking k_D to the potential of mean force requires making assumptions in order to account for hydrodynamic forces,

and different theoretical expressions are obtained depending on the definition of the diffusion coefficient at the molecular level.[100–103] A number of studies have applied these relationships for relating k_D to a potential of mean force model and obtained similar fit parameters to those obtained from B_{22} studies[111,115,116] indicating that k_D can be used as a surrogate for B_{22}. In addition, there is a linear correlation between the two parameters that has been demonstrated to hold true for a range of different antibodies.[6,15,111,114] While this correlation has been tested for different proteins, the effects of using different salts has not yet been investigated. The results of B_{22} studies for solutions at pH 5 containing the salts NaCl, NaSCN, Na_2SO_4, or $CaCl_2$ are shown in Figure 2a. The contribution from the acetate buffer to the ionic strength was set at 25 mM in all the experiments. In Figure 2b is shown the correlation between the measured B_{22} values and the interaction parameter k_D measured for the same solvent conditions. In the current work, we are interested in the effect of changing salt type on k_D for solutions at the same ionic strentgh and pH. For understanding these changes we only require that there exists a monotonic relationship between B_{22} and k_D. In a previous study, we showed that the correlation can be rationalized using an approximate relationship between the value of k_D and the protein potential of mean force.[111] A useful reference point for any protein-protein interaction measurement is the excluded volume term, which is given by $B_{22} = 6.7 \times 10^{-4}$ mL-mol/g^2 or $k_D = 3.8$ mL/g.[111,121] Any value greater than the excluded volume term, indicates the presence of a longer-ranged protein-protein repulsion, whereas a more negative value reflects a protein-protein attraction. For the mAb1, a weak protein-protein attraction is observed for all solution conditions except for at low ionic strength and low pH where electrical double layer forces dominate the behaviour.

Values of k_D obtained in solutions of mAb1 plotted as a function of ionic strength for solutions at pH 5 or 6.5 are shown in Figure 3a and b. In solutions at pH 5, the trends with ionic strength and salt type are the same as observed from the B_{22} studies. At low ionic strength, the interactions in sodium sulfate solutions are less repulsive than in solutions with sodium chloride as the background salt. With increasing ionic strength, the difference is reduced sufficiently such that, within experimental error, the interactions are the same for sulfate or chloride containing so-

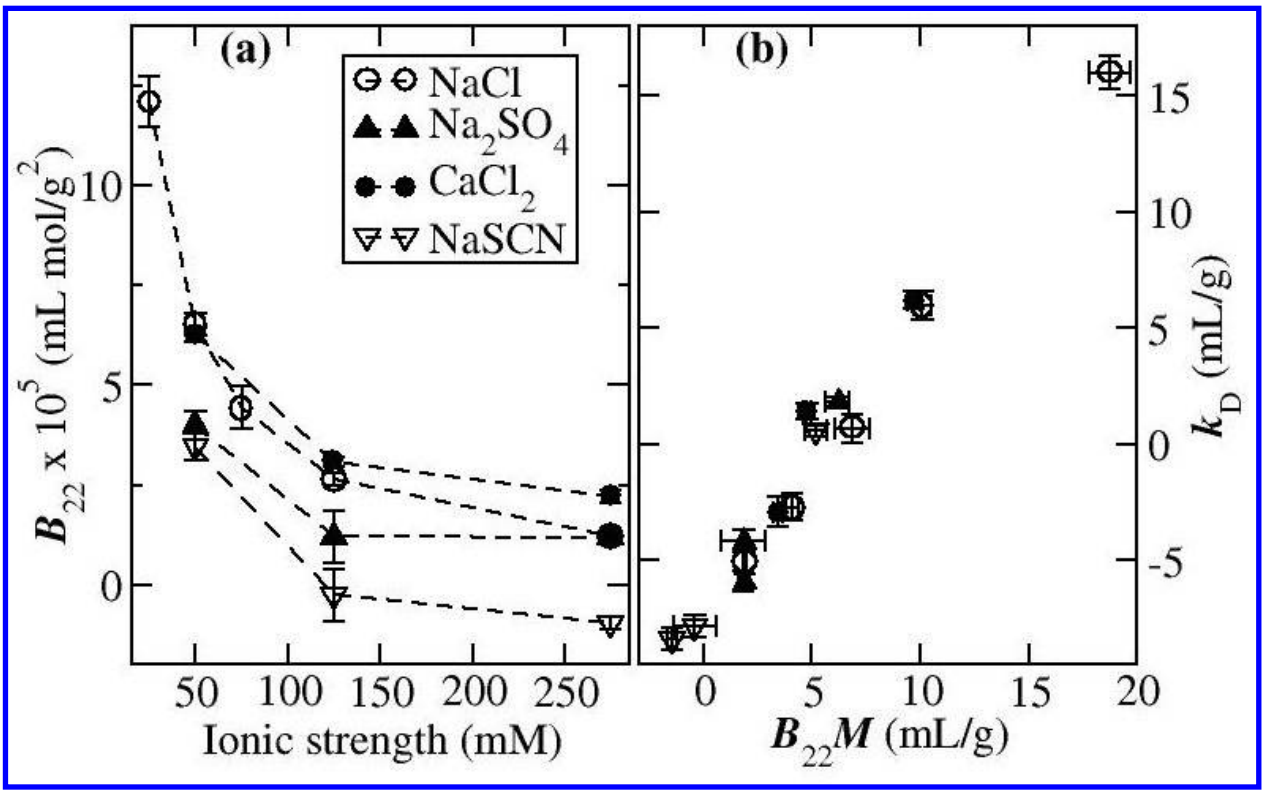


Figure 2: **(a)** B_{22} values measured as a function of ionic strength for solutions at pH 5 containing either sodium chlroide, sodium sulfate, sodium thiocyanate, or calcium chloride. **(b)** The correlation between values of B_{22} and k_D obtained for the various salt solutions shown in **(a)**. An acetate buffer has been used in all measurements at an ionic strength contribution of 25 mM.

lutions at ionic strengths of 275 mM or 525 mM. For both pH values, the interactions are made more repulsive when switching from sodium to calcium at fixed ionic strength, except in comparisons at an ionic strength of 50 mM. The interaction patterns in sodium thiocyanate solutions are slightly more complicated exhibiting a non-montonic depedence on ionic strength, with the minimum shifted to lower ionic strength with increasing pH. At low ionic strength, the greatest amount of protein-protein attraction is observed in sodium thiocyanate solutions versus all other salts. A salt-induced repulsion is exhibited at moderate ionic strength (200 mM) and leads to larger values of k_D than for any other salts at pH 6.5 and an ionic strength of 525 mM.

In Figure 4 is shown the k_D measurements for mAb1 in solutions at pH 8 or 9, where the contribution of tris buffer to the ionic strength is 5 mM and 10 mM, respectively. The remaining ionic strength is made up of the corresponding salt solution. Out of the low ionic strength con-

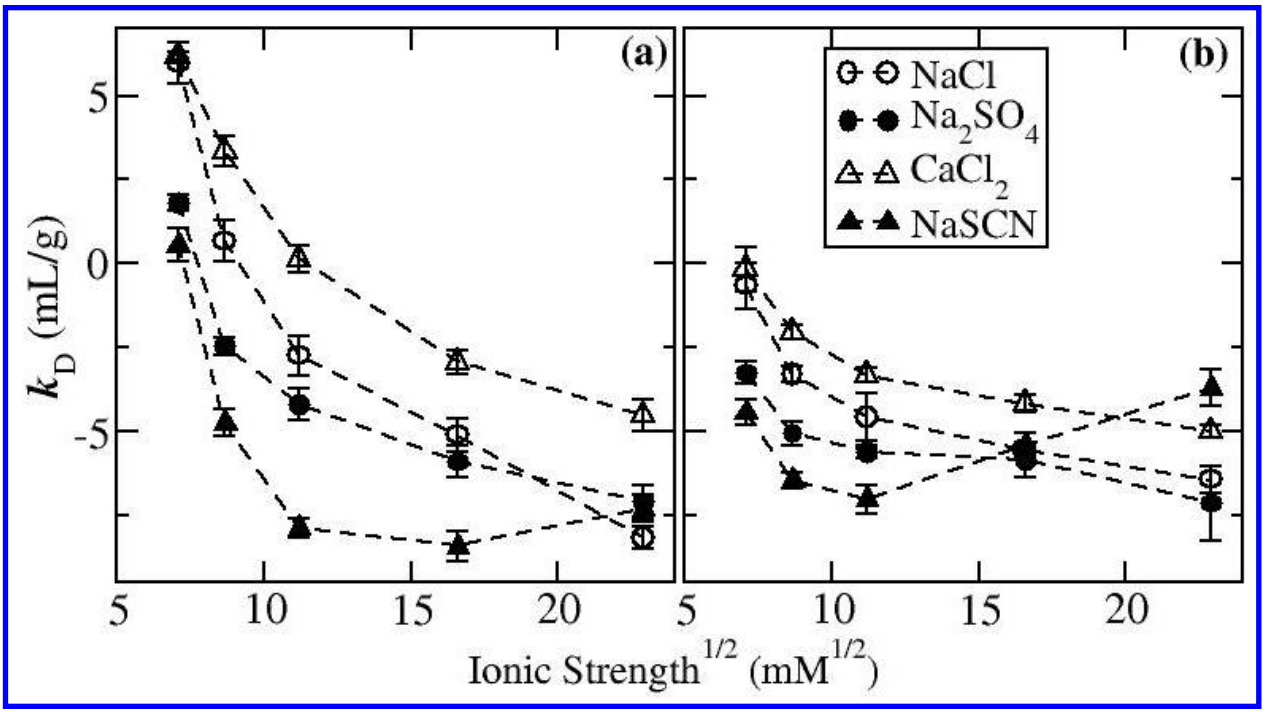


Figure 3: Plots of k_D versus ionic strength in salt solutions containing either NaCl, $CaCl_2$, Na_2SO_4, or NaSCN at **(a)** pH 5 with 25 mM acetate and **(b)** pH 6.5 with 25 mM acetate. The buffer concentration corresponds to the contribution of the buffer species to the ionic strength of the solution

ditions investigated here, the protein-protein interactions are most attractive in solutions at pH 9. As mentioned previously, the attraction has an electrostatic origin as it is reduced by increasing the ionic strength. A similar trend, but to a lesser extent, is also observed with solutions at pH 8 containing either NaSCN or Na_2SO_4. In solutions at low ionic strength, only a small specific anion effect is observed at pH 8 and no specificity occurs at pH 9. At higher ionic strength, however, there is a large anion-specific salting-in effect observed with thiocyanate as inferred from the increase in protein-protein repulsion relative to the other sodium salts. In solutions at pH 8, the effect of changing from sodium to calcium is to increase the protein-protein repulsion over all ionic strength, whereas this increase is only observed in low ionic strength solutions at pH 9. The non-monotonic protein-protein interaction patterns observed with respect to ionic strength at pH 9 can not be rationalized using electrostatic approaches. In particular, it is not clear why there is a salt-induced attraction when increasing ionic strength from 275 mM to 525 mM.

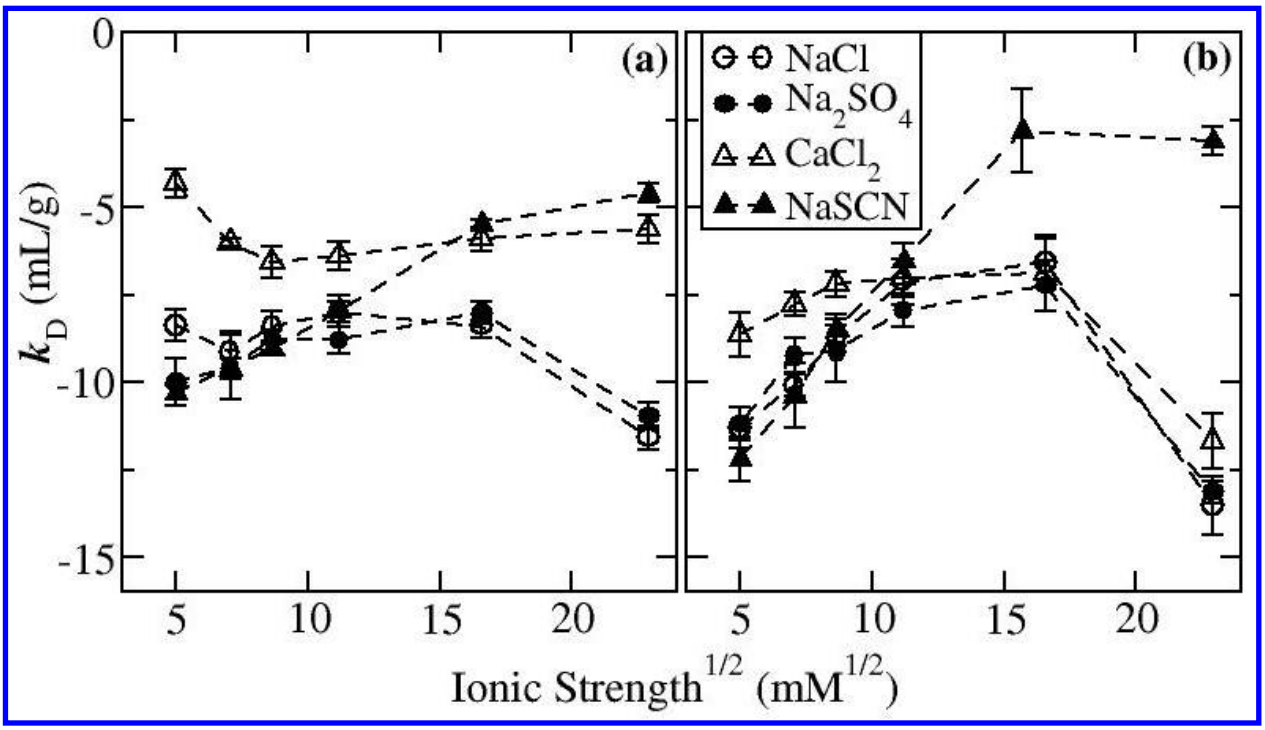


Figure 4: Plots of k_D versus ionic strength in salt solutions containing either NaCl, $CaCl_2$, Na_2SO_4, or NaSCN at **(a)** pH 8 with 10 mM tris and **(b)** pH 9 with 5 mM tris. The buffer concentration corresponds to the contribution of the buffer species to the ionic strength of the solution

Anion specific effects

Comparing the results for sodium sulfate versus sodium chloride provides insight into how ion binding alters protein-protein interactions. Within DLVO theory, changing salt concentration can affect either the screening length of the solution or alter the protein charge via ion binding. The key difference is that screening is a non-specific salt effect which is determined by the ionic strength of the salt solution, whereas ion binding follows saturation behaviour characterized by ion-specific binding constants. Sodium chloride has previously been used as a model salt for investigating the effect of screening on protein or peptide conformational stability.[68] However, a chloride binding dissociation constant on the order of 150 mM was estimated from the effect of chloride salts on the thermal stability of ubiquitin at low pH.[61] Electrophoretic mobility measurements were also used to deduce that RNase A binds two chloride ions in 100 mM potassium chloride solution.[122] Thus while we have previously interpreted the effect of increasing sodium chloride on protein-

protein interactions in terms of ionic screening, it is likely that there is also a minor effect due to charge neutralization. These effects can be disentangled from each other using measurements in solutions containing sulfate, which exhibits much stronger binding to protein charged groups. For instance, the salt concentration at the mid-point of the acid-induced refolding tranisition for either cytochrome C or apomyoglobin was found to be 1.25 mM or 2.5 mM for sodium sulfate versus 45 mM or 75 mM for sodium chloride solutions.[63] At pH 2, the positively charged state of RNAse A is stabilized against thermal unfolding in low ionic strength solutions containing sodium sulfate, but no stabilization occurs in sodium chloride indicating the sulfate effect is only due to anion binding and not charge screening.[62] Furthermore, the study found that the sulfate binding effect is saturated by a salt concentration of 100 mM. The much stronger binding of sulfate is also reflected by salt effects on conformational transitions of charged homopolypeptides. In particular, co-polymers made of leucine and lysine undergo a transition to either an α-helical or β-sheet state upon charge neutralization through anion binding. The transition occurs at concentrations as low as 3 mM for sulfate-containing salts, whereas no transition is observed at salt concentrations up to 100 mM for salts of fluoride or chloride.[70,123] Thus we expect sulfate binding to mAb1 will be saturated in solutions at the low ionic strength values investigated here. This is consistent with the experimental findings presented in Figure 3 for pH 5 and 6.5. Protein-protein interactions are less repulsive in sodium sulfate versus sodium chloride solutions at the same ionic strength due to sulfate binding and charge neutralization. The effect of sulfate binding is greatest at low ionic strength, conditions where the electrostatic forces are largest. Because sulfate binding is saturated at low salt concentration, the primary effect of increasing ionic strength is to screen electrostatic interactions such that the effect of sulfate neutralization is reduced and becomes negligible at 275 mM ionic strength. At this same ionic strength, there is also very little effect of varying pH from 5 to 9 in sodium chloride solutions (see Figure 1) providing further evidence that electrostatic effects are screened at an ionic strength of 275 mM.

Next we compare the behaviour of sodium thiocyanate to either sodium chloride or sodium sulfate. Both thiocyanate and sulfate anions have strong affinities for positively charged protein

groups, but in contrast to sulfate, thoicyanate also preferentially interacts with uncharged protein surface groups. At low ionic strength and acidic pH, the strongest protein-protein attraction is observed in sodium thiocyanate solutions. However, at high ionic strength protein-protein interactions are more repulsive in solutions of sodium thiocyanate versus any other sodium salt. A similar pattern of protein-protein interactions with respect to anion type occurs for lysozyme as deduced from UCPT studies.[35] At low salt concentration lysozyme solubility follows the reverse Hofmeister series due to the ability of chaotropic anions to neutralize positively charged protein groups and lower the electrical double layer forces, which is reflected by the effect of sodium thiocyanate on the k_D values for the mAB1 at low ionic strength. At higher salt concentrations, lysozyme solubility is inversely related to the protein-solution interfacial tension. Due to the stronger preferential adsorption of chaotropic anions to proteins, the interfacial tension decreases with the descending position of the anion in the Hofmeister series leading to the direct Hofmeister dependence of lysozyme solubility. The latter "salting-in" effect is manifested by the thiocyanate-induced repulsion at high ionic strength for mAb1.

The meaured k_D values for mAb1 in thiocyanate indicate more effective charge neutralization and reduced electrical double layer forces when compared with either sulfate or chloride. Unlike what happens with sulfate solutions, the difference in k_D for solutions of sodium thiocyanate versus sodium chloride initially increases with rising ionic strength. The implication is that the binding of thiocyanate ions to mAb1 is not saturated at such low salt concentration as occurs with sodium sulfate. When compared at the same ionic strength, solutions of sodium sulfate are equally as effective as sodium thiocyanate at inducing the acid-induced refolding transition of cytochrome C indicating similar saturation behaviour for both anions.[63] The refolding studies only reflect the site binding of thiocyanate to positively charged groups, whereas the double layer forces are determined by the average diffuse layer potential, which is also controlled by the preferential adsorption of thiocyanate to the uncharged parts of mAb1. While preferential adsorption leads to salt-induced repulsion at moderate to high ionic strength, in low ionic strength solutions, the adsorption will lower the surface potential and further reduce double layer forces. Thus, the enhanced electrostatic

screening observed in sodium thiocyanate solutions is due to the weak preferential adsorption of thiocyanate, rather than ion pair formation and charge neutralization.[35] This reasoning is consistent with recent theoretical and simulation studies, which have examined the effect of ion specificity on the electrical double layer potential and the link to protein-protein interactions.[124–127] The preferential adsorption of chaotropic anions has been captured by incorporating ion-surface dispersion forces self consistently in the Poisson Boltzmann equation to describe the double layer about a model surface mimicing the charged state of lysozyme.[124,126] Increased preferential adsorption to the surface occurs for chaotropic anions due to their large excess polarizabilities. Adsorption screens the protein surface charge thereby lowering the ion concentration in the double layer and reducing the osmotic repulsion force, an effect which correctly captures the reverse Hofmeister series dependence of solubility at low ionic strength. Further increasing salt concentration leads to more adsorption and a change in the surface charge from positive to negative. The negative surface charge draws cations into the double layer increasing the ion double layer concentration and creating an osmotic repulsion force. This effect then leads to a reversal of the Hofmeister series at high salt concentration as preferential adsorption is correlated with increased protein-protein repulsion.

For sodium thiocyanate solutions, protein-protein interactions over the ionic strength range of 125 to 525 mM at pH 5 are more attractive than any of the protein-protein interactions measured at pH 6.5. Rationalizing this behaviour in terms of how ion binding alters double layer forces is not possible. The minimum in each of the curves should correspond to when the double layer force or surface potential goes to zero due to neutralizing the protein charge. As expected, the minimum occurs at a lower ionic strength when pH is increased as less ion binding is needed to compensate the lowered positive charge on the protein. However, in order to explain the smaller B_{22} values at pH 5 requires invoking the existance of an additional salt-dependent protein-protein attraction, which must also exist at an ionic strength of 275 mM. Here, the effect of altering the protein ionizable charge is screened providing additional evidence that the thiocyanate effect can not be rationalized by a charge neutralization mechanism. Similar interaction patterns for another monoclonal antibody can be deduced from specific ion effects on UCPT studies made as a function

of pH and ionic strength.[11] In particular, a maximum UCPT with respect to ionic strength occurs around 100 mM in solutions at pH 5.3 with potassium thiocyanate. The ionic strength at the maximum shifts to lower values with increasing pH, which follows the same protein-protein interaction pattern that occurs with mAb1.

Cation specific effects

The cation specific effects become apparent when comparing the k_D values for solutions of calcium chloride versus sodium chloride shown in Figure 3 and Figure 4. Analogous to the discussion of anion specific effects, in low ionic strength solutions, cation specificity arises from differential ion binding affinity to negatively charged protein groups. The relative ion pair affinities of divalent versus monovalent cations have been estimated from the effect of salt concentration on the UCPT of an elastin-like polypeptide containing aspartic acid groups.[84] The measured K_d values for divalent cations with the carboxylate group ranging from 1 to 10 mM reflect a much stronger affinity than occurs with monovalent cations, which have K_d values on the order of 80 to 350 mM. The strong association of divalent cations to negatively charged proteins leads to a signficant reduction in the surface potential as characterized from electrophoretic mobility measurements,[128–130] and leads to a reduction in double layer forces in low ionic strength solutions.[131] In the solutions at pH 8 and at pH 9, mAb1 carries a slight positive potential as determined from ζ-potential measurements in solutions containing 10 mM NaCl.[111] Under these conditions, calcium binding will overcharge mAb1 leading to an increase in the double-layer forces relative to sodium chloride at low ionic strength. In contrast, for solutions at pH 5 or 6.5, the calcium induced repulsion becomes apparent at an ionic strength of 75 mM. The main effect of changing pH from 8 to 6.5 is the protonation of histidine groups leading to a larger net positive charge on mAb1. At low ionic strength, the electrostatic repulsion between calcium and the net protein positive charge prevents calcium from binding to the protein. However, with increasing ionic strength, the screening length is decreased such that calcium binding interactions are localized and the repulsive electrostatic interactions with nearby positive groups become shielded.[41,132] As a consequence, the enhancement of double layer

forces only becomes apparent with increasing ionic strength at lower pH values. While we have not characterized the binding of calcium to the protein surface in this study, the binding of magnesium to another mAb at a pH of 4.8 was reflected by a slightly more positive ζ-potential versus solutions of sodium chloride at the same ionic strength. The differences in binding affinities of calcium and magnesium are small when compared to the changes expected from switching between a divalent and a monovalent cation.[84] While calcium binds to mAb1 at moderate pH, we expect further lowering pH will have the opposite effect as carboxylate groups become protonated leading to a reduction in calcium binding sites. In acidic conditions, divalent cations are excluded about the protein due to electrostatic interactions leading to a lower positive potential in divalent versus monovalent cation salts.[130,133]

The effects of calcium can not only be rationalized in terms of changes to double layer forces because the cation specific effects are still significant at ionic strengths up to 575 mM for solutions with pH ranging from 5 to 8. These salting-in effects of divalent cations at moderate ionic strengths have been previously quantified from direct protein-protein interaction measurements in terms of B_{22}[34,134] or k_D,[135,136] from solubility and LCPT measurements,[26–28,41,42] from the resolubilization of protein coated particles,[129,133,137] and from AFM measuremnts of the force profile between protein coated particles.[138,139] The salting-in effect results from calcium adsorption to either polar or charged protein groups. The most likely polar cation binding site is the carbonyl oxygen along the protein backbone. However, a spectrocopic study of the interactions between the carbonyl oxygen of butyramide and cations indicated very little difference in the affinity towards divalent versus monovalent cations. The binding of divalent cations is extremely weak with a K_D equal to approximately 10 M, whereas binding of monovalent cations could not be detected.[90] Because cation binding to carboxylates is stronger and much more sensitive to the nature of the cation has led to the suggestion that cation specificity on salting-in behaviour is predominantly determined by ion pair formation.[84,87,91,140] In this case, the salting-in effects observed at ionic strengths above 275 mM need to be rationalized in terms of non-DLVO type repulsion between proteins complexed with divalent cations. One possible mechanism is a repulsive hydration forces which arises from

the energy needed to dehydrate hydrophilic or charged surfaces as they approach each other.[133,138] These types of forces have been extensively characterized between negatively charged surfaces in electrolyte solutions, where the magnitude and range of the force increase with the hydration number of the bound cations.[141] As such, the force becomes more repulsive when increasing the valency of the cation reflecting the increase in hydration number. An AFM study of the interaction between particles coated with either BSA or ferritin confirmed the presence of the hydration force from the inter-particle force profile observed in moderate ionic strength solution, which was more repulsive in calicum chloride versus sodium chloride.[138,139] A similar explanation has been invoked to explain a commonly-observed increase in protein-protein attraction for globular proteins when reducing pH at high ionic strength.[142,143] In this case, the attraction is attributed to removing the hydration force when carboxylate groups become protonated in acidic solutions.

Alternatively, the salting-in power of calcium could also be linked to the preferential adsorption to the polar protein surface.[144,145] Protein-salt preferential interaction parameter measurements of salts with divalent cations reflect a preferential adsorption mechanism as the salt is less excluded about BSA than occurs with sodium sulfate or sodium chloride.[26–28] Interactions with charged groups are saturated at low salt concentration and do not contribute to the preferential interaction parameter measurements at salt concentrations of 0.5 M or higher. Thus, the weak exclusion of divalent cation salts about proteins is due to interactions with the polar protein surface, which is consistent with the effect of divalent cations on the aqueous solubility of capped peptides and conformational transitions of uncharged polypeptides.[49,51,54,146] The impact of weak preferential adsorption on protein-protein interactions is reflected by the comparison in k_D for mAb1 when changing from sodium chloride to sodium thiocyanate at alkaline pH. The thiocyanate induced protein-protein repulsion reflects the effect of salt adsorption to the protein surface. A similar increase in protein-protein repulsion is observed when changing from sodium chloride to calcium chloride. In this case, the mechanism for the salting-in ability is not necessarily related to hydration forces of the adsorbed species since thiocyanate is weakly hydrated, yet effective at salting-in. Instead the repulsion might be due to an osmotic repulsion force that arises when the regions of

solvent perturbation around proteins overlap with each other. When salts preferenially adsorb to protein surfaces, there will be an increase in the local osmotic pressure due to the higher concentration of ions relative to in the bulk solution. A similar mechanism has already been used to describe the opposite behaviour observed during salting-out processes. In that case, the protein-protein attraction arises due to an osmotic depletion force as the salt ions are exclued from between the proteins relative to the bulk.[31]

Specific buffer effects

Next, we examined the ionic strength dependence of protein-protein interactions in solutions containing the buffers histidine, acetate, succinate, phosphate, or citrate for solutions at pH 5 and 6.5. The inital aim was to probe the effect of buffer binding on protein-protein electrostatic interactions, which requires comparing measurements at fixed ionic strength such that the screening length is the same. As such, the contribution of the buffer salt to the ionic strength has been fixed at 25 mM, with the only exception being histidine buffer at pH 6.5, where we set the ionic strength contribution of the buffer to 10 mM. The remaining ionic strength was made up with sodium chloride, which behaves predominantly as a screening salt. Results of the k_D measurements are shown in Figure 5. At an ionic strength of 25 mM, there is a large dependence of k_D on the buffer type, which reflects the effects of buffer binding on the protein electrostatic properties. All buffer ions except histidine are negatively charged, in which case, buffer binding reduces the protein surface potential leading to less double layer repulsion. The effectiveness of the buffer at reducing the repulsion in solutions at pH 5 is given by citrate $>$ succinate $>$ phosphate $>$ histidine $\sim$ acetate. At pH 6.5, the effectiveness of the ions follows approximately the same order, citrate $>$ succinate $\sim$ phosphate $>$ histidine $\sim$ acetate. With increasing ionic strength, the effect of buffer becomes almost unnoticeable at 125 mM ionic strength, and in solutions at 275 mM ionic strength, the k_D vales are similar for both pH values and all types of buffers. The finding that protein-protein interactions are independent of buffer at an ionic strength of 275 mM provides direct evidence that the only effect of buffer binding is to alter protein electrostatic interactions. The effects are screened

at the same ionic strength that is found to screen the effect of pH on protein-protein interactions (see Figure 1). The trends observed here are similar to those determined for other monoclonal antibodies in k_D studies using sodium buffers at a fixed concentration of 50 mM at pH 5.0,[7] a B_{22} study as a function of buffer concentration at pH 6.2,[107] and a study where protein-protein interactions were inferred from a PEG-precipitation assay with buffer concentrations fixed at 50 mM as a function of pH.[106] The similarities in these studies indicate that the mechanism for buffer effects is non-specific and will be manifested in other antibody systems.

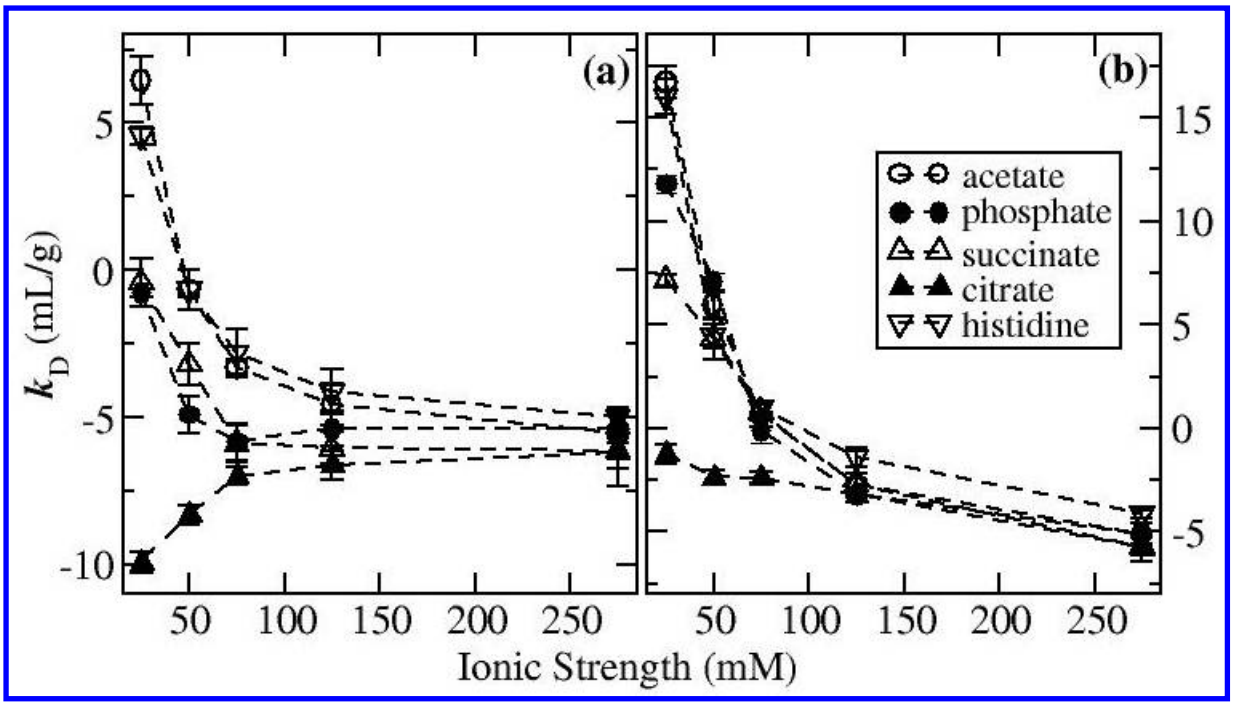


Figure 5: Plots of k_D as a function of ionic strength for solutions containing either histidine, acetate, phosphate, succinate, or citrate buffers at **(a)** pH 6.5 and **(b)** pH 5. The ionic strength contribution of all buffer salts is equal to 25 mM, except for histidine chloride at pH 6.5 which is equal to 10 mM. NaCl was used to adjust the total ionic strength of all solutions.

Buffer binding to the protein surface was further probed by calculating the ζ-potential from electrophoretic mobility measurements for the solutions at 25 mM ionic strength. For a uniformly-charged spherical particle, the ζ-potential corresponds to the electrical potential at a fictitious slip plane, which separates the bound from flowing solvent.[147] The slip plane is located just outside the edge of the stern layer. In this case, the ζ-potential value will provide a useful estimate for the

double layer potential, which in turn, reflects the protein fixed charge including ions contained in the stern layer. For surface potentials less than 50 mV, the polarization of the double layer under the applied electric field is negligible. In this case, the Henry equation can be used to relate the measured electrophoretic mobility μ_E to ζ-potential

$$\zeta = \frac{3\eta}{2\varepsilon_0 \varepsilon f(\kappa a)} \mu_E \tag{8}$$

where a is the particle radius, ε_0 is the vacuum permitivity, ε is the dielectric constant of the medium, and η is the solution viscosity. $f(\kappa a)$ is the Henry's function, which can be approximated by

$$f(\kappa a) = 1 + \frac{1}{2}\left[1 + \left(\frac{2.5}{\kappa a[1 + 2\exp(-\kappa a)]}\right)\right]^{-3}. \tag{9}$$

For an antibody, the measured ζ-potential appears to underestimate the diffuse layer potential when assuming a spherical geometry and uniform charge distribution for the antibody.[22,111,114] In those studies, antibody charge estimates from ζ-potential measurements were much less than expected from potentiometric titrations or calculated charges from the amino acid sequence. Nevertheless, despite these inconsistencies, an increase (or decrease) in the ζ-potential measurement will correlate with an increase (or decrease) in the electric double layer potential as long as trends are compared at the same ionic strength.[148] Here, we also report an effective charge that corresponds to the charge of a sphere with the same hydrodynamic radius and electrophoretic mobility as the antibody. The charge can be calculated by solving the Poisson-Boltzmann equation using the Debye-Huckel approximation to give

$$eZ_\zeta = \frac{6\pi\eta a}{f(\kappa a)}(1 + \kappa a), \tag{10}$$

where e is the electronic charge equal (1.602 x10$^-$19 C).

The ζ-potentials measured for solutions at pH 5, 6.5, 8, or 9 are shown in Figure 6 along with the effective charge Z_ζ calculated using Eq. (10) where the measured value of $R_h = 5.15$

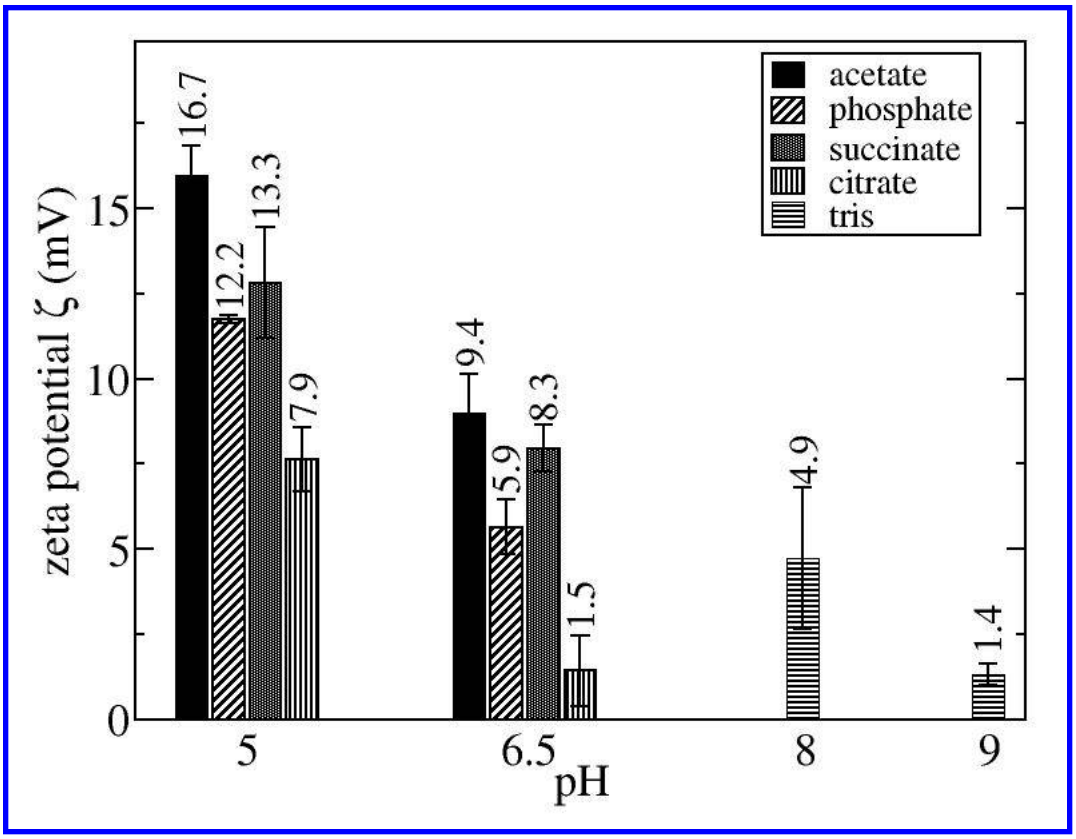


Figure 6: Zeta potential measured for different buffers at an ionic strength of 25 mM. Numbers located above the bars correspond to the value for Z_ζ

nm is used as the radius a in Eq. (10). All the measured potentials are less than 20 mV and correspond to conditions where Henry's equation (Eq. (8)) is valid. The values reported here for Z_ζ at pH 6.5 are in the same range as reported in other ζ-potential studies of antibodies at pH 6 in histidine-hydrochloride buffer at ionic strength between 15 and 20 mM.[22,114] At pH 5 and 6.5, the effectiveness of the buffer at reducing the protein charge follows the order citrate $>$ phosphate $>$ succinate $>$ acetate. The results follow what was expected from the k_D measurements as reduced protein-protein repulsion is correlated with the buffer binding to the protein surface. The slight exception to this rule occurs when comparing phosphate to succinate as the interactions are slightly more repulsive in phosphate solutions at pH 5. Since citrate, succinate, and phosphate all exist as multivalent anions in solution, it is tempting to rationalize the binding affinities in terms of electrostatic interactions which are proportional to the negative charge carried by the buffer

species. At pH 5, the averaged net charges of citrate (pK_a = 3.1, 4.8, 6.4), succinate (pK_a = 4.2, 5.6), and phosphate (pK_a = 2.1, 7.2, 12.7) are approximately 2, 1.5, and 1, respectively, which follows roughly what is expected from the ζ-potential studies. Non-electrostatic interactions also control ion binding specificity, which is best examplified comparing the behaviour of sulfate to citrate. In solutions at pH 5 and 50 mM ionic strength, the averaged net charge of citrate is close to 2, however, citrate is much more effective at attenuating the double layer repulsion than sulfate.

The ζ-potential measurements correlate well with the k_D values when comparing the solutions of citrate at pH 5 and 6.5 with the alkaline solutions at pH 8 and 9, respectively. Attractive protein-protein interactions are greatest in solutions at pH 9 and in solutions containing 25 mM citrate buffer at pH 6.5 according to the k_D measurements; these conditions also correspond to a ζ-potential that is close to zero. Analogous to the behaviour at pH 9, the ionic strength trend of k_D indicates the presence of attractive electrostatic interactions in citrate buffer at pH 6.5. Because citrate is multivalent at pH 6.5, binding to monovalent charges on the protein surface will lead to charge inversion and the formation of negatively charged patches. A distribution of positive and negative charge on the protein surface is a prerequisite for the attractive electrostatic interactions. Charge inversion has been observed with negatively charged proteins in solutions of salts containing trivalent and divalent cations.[131,149,150] The ability of multivalent carboxylic acids to cross-link proteins together has been inferred previously from their ability to promote protein crystallization.[2,3] The finding that the cross-linking interactions are screened by salt implies that the beneficial effects of these molecules will only be observed in low ionic strength crystallization screens.

Because the effective charge on the protein determines the magnitude of the protein-protein repulsion at low ionic strength, k_D measurements can be used as an indirect method for assessing ion binding. In order to infer ion binding from k_D measurements requires comparing values at fixed ionic strength such that the effect of ionic screening on electrostatic interactions is the same. In Figure 7a and Figure 7b are shown values of k_D for mAb1 dissolved in pH 5 solutions at different concentrations of either phosphate buffer or citrate buffer along with sodium chloride to control

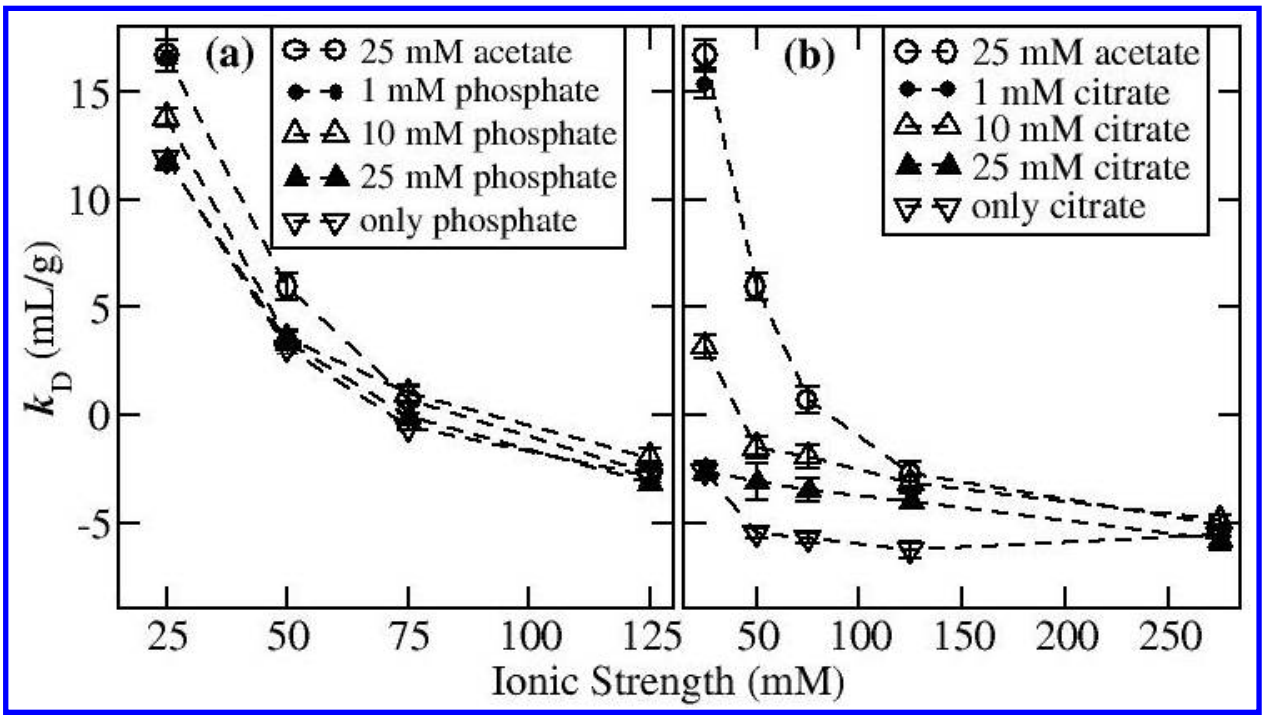


Figure 7: Plots of k_D versus ionic strength in salt solutions at pH 5 containing **(a)** 25 mM acetate, 1 mM phosphate, 10 mM phosphate, 25 mM phosphate, and only phosphate buffer and **(b)** 25 mM acetate, 1 mM citrate, 10 mM citrate, 25 mM citrate, and only ctrate buffer. Sodium chloride was used to adjust the ionic strength of the solutions in all cases except for 1 mM buffer solutions in which case a 10 mM acetate buffer was also used

the ionic strength. In the solutions of phosphate buffer, no effect of buffer is observed at a buffer concentration of 1 mM (which corresonds to 1 mM ionic strength contribution from sodium phosphate). The impact of buffer binding is observed when increasing ionic strength of the buffer to 10 mM and then to 25 mM, which leads to a significant reduction in the protein-protein repulsion. However, for solutions containing 25 mM phosphate buffer, increasing ionic strength by adding sodium phosphate has the same effect as adding sodium chlroide. This indicates that the primary effect of inceasing ionic strength above 25 mM is to screen electrostatic interactions; if ion binding was significant, then we would expect to see a difference in the k_D values at ionic strengths greater than 25 mM.

The effects on protein-protein interactions of citrate versus phosphate binding are much more pronounced. Citrate has a stronger binding affinity than phosphate as reflected by a small reduction

in k_D at low citrate concentrations. An ionic strength contribution from citrate buffer of 1 mM is equivalent to a buffer concentration of ~0.4 mM. The behaviour is consistent with what has been observed from isothermal titration calorimetry measurements that estimated a dissociation constant K_d for citrate binding by a mAb on the order of 1 mM in solutions at pH 5.[151] The citrate buffer binding is not yet saturated in solutions with an ionic strength of 25 mM. The k_D values observed in solutions with 50 mM ionic strength where only citrate is used as the anion, are less than the corresponding ones with a mixture of sodium chloride and sodium citrate. At this ionic strength, the net contribution of electrostatic forces is very small as the k_D value is similar to the value obtained at an ionic strength of 275 mM. The value of k_D in a solution of sodium citrate at 275 mM ionic strength is similar to the value obtained for 250 mM NaCl and 25 mM acetate buffer indicating the effect of citrate binding is to only alter electrostatic interactions between proteins.

We have also determined that there is no observable effect on protein-protein interactions when comparing a buffer containing only sodium acetate at an ionic strength of 25 mM to a buffer of the same ionic strength composed of sodium acetate and sodium chloride at a concentration of 15 mM. The similar effects of chloride and acetate reflects their positions in the middle of the Hofmeister series. In Figure 8a and b are shown comparisons of protein-protein interactions with varying amounts of histidine buffer in solutions at pH 6.5 and with varying the buffer type and concentration in solutions at pH 8, respectively. Sodium chloride is used as the background electrolyte in all cases. There is a systematic decrease in k_D for all ionic strengths when increasing the amount of histidine in the buffer. Ionic strength contributions from histidine buffer of 10 and 25 mM correspond to buffer concentrations of approximately 40 and 100 mM, respectively. The effect of histidine on protein-protein interactions has a non-electrostatic origin as there is a small change in k_D at high ionic strength. The constant offset in the k_D curve when changing the histidine buffer composition is similar to the effect on k_D when changing sodium chloride to calcium chloride. Calcium induced repulsion has been attributed to protein-protein hydration forces originating from binding calcium to the protein. Similarly, sodium ions can be displaced from the protein surface by increasing concentrations of histidine. This in turn could lead to a

reduction in repulsive hydration forces, which have been observed in sodium chloride solutions, but to a lesser extent than with calcium chloride. In the solutions at pH 8, within the experimental error, there is no effect of changing the composition of the buffer. This is not surprising in that no effect of sulfate or thiocyanate was also observed in solutions at pH 8, although, both of these anions bind to the positively charged protein groups.

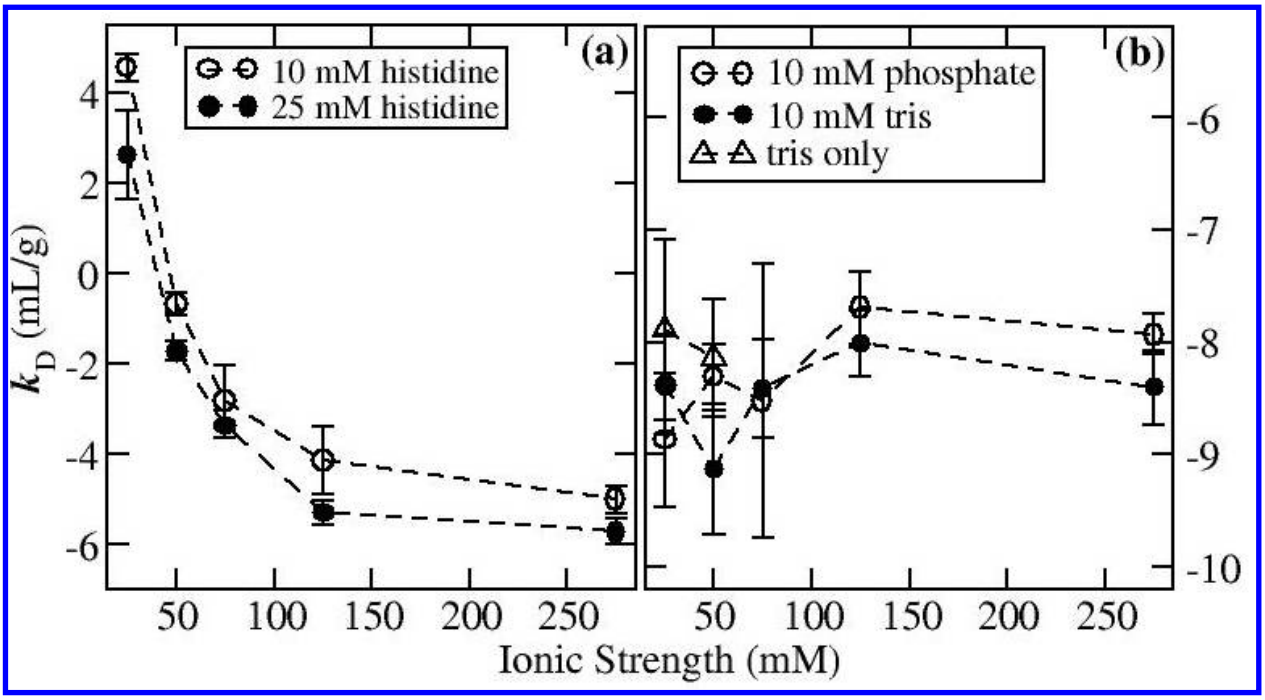


Figure 8: Plots of k_D versus ionic strength for the mAb1 in sodium chloride solutions **(a)** at pH 6.5 containing 10 mM or 25 mM histidine buffer and **(b)** at pH 8 containing 10 mM phosphate, 10 mM tris, or tris buffer only.

Conclusions

The main purpose of this investigation into protein-protein interactions was to separate out the different salt effects, including ion binding verus ionic screening, as well as salting-in and salting out interactions. Ion binding and screening impact on protein electrostatic interactions, which dominate in low ionic strength solutions. We have shown that the upper limit to this range occurs around an ionic strength of 275 mM, conditions where protein-protein interactions are independent

of pH indicating electrostatic effects are sufficiently screened. The effects of ionic sreening and ion binding can be delineated by comparing solutions containing sulfate to those with chloride at the same ionic strength. Sulfate ion binding neutralizes some of the protein charge leading to reduced double layer forces when compared with sodium chloride. Sulfate binding is already saturated by the lowest ionic strength condition investigated here. As such, increasing sodium sulfate concentration only screens electrostatic interactions as reflected by the decreasing difference in k_D values when compared to sodium chloride solutions at the same ionic strength.

The behaviour contrasts with what happens in sodium thiocyanate solutions at low pH. Although, sulfate and thiocyanate exhibit similar affinities to protein charged groups,[63] the difference in k_D with respect to sodium chloride is not screened implying that thiocyanate binding, unlike sulfate, is not saturated in the low ionic strength solutions. At higher sodium thiocyanate concentrations, the salting-in effect dominates as reflected by an enhanced protein-protein repulsion relative to sodium chloride solutions. Salting-in effects are due to salt preferential adsorption to uncharged protein regions. The salt-induced repulsion however only becomes apparent at higher salt concentration where electostatic interactions are screened. In low ionic strength solutions, weak anion adsorption to basic proteins will enhance the screening of electrostatic interactions, which explains why thiocyanate is more effective than sulfate at reducing protein-protein repulsion at low pH. The competition with salting-in leads to a non-monotonic dependence of protein-protein interactions, which is most pronounced for mAb1 in solutions at pH 6.5. This behaviour has been captured using theoretical approaches, where increasing salt concentration initially decreases the double layer potential due to preferential anion adsorption until reaching a critical salt concentration where the double layer potential goes to zero.[124–127] Further increasing salt concentrations reverses the surface potential leading to the salting-in behvaviour. This view is consistent with the finding that the minimum in the protein-protein attraction shifts to higher ionic strength when pH is lowered as more salt is needed to balance the increased positive charge of the protein. Similar patterns of protein-protein interactions have also been observed in the UCPT studies of another monoclonal antibody in thiocyanate solutions.[11] However, the increased protein-protein attraction

observed in thiocyanate solutions when lowering pH from 6.5 to 5 at ionic strengths of 275 mM can not only be rationalized based on an electrostatic mechanism. One possibile explanation is that the increased number of thiocyanate binding sites at low pH can lead to large fluctuations in the charge distribution. These can give rise to an attractive charge regulation interaction between proteins.[152] Alternatively, increased binding of thiocyanate anion can alter the nature of short ranged forces between proteins. For instance, thiocyanate binding to antibodies has been linked to increased local flexibility in specific regions of the protein, which could indirectly lead to an effective protein-protein attraction from exposing sticky hydrophobic groups for instance.[153] Or yet, another distinct possibility, is that the thiocyanate-specific effect is due to a change in the anisotropic nature of the protein-protein interaction when reducing pH from 6.5 to 5.[97,99]

Acknowledgements

Dorota Roberts was supported by a BRIC/BBSRC grant number BB/I017194/1. Robin Curtis had an ISS grant from the Royal Academy of Engineering that supported his part-time placement in 2013 at MedImmune. The authors thank Wyatt Technology for continued instrument support and helpful discussions, in particular, Kevin Jackson, Dan Some, and Sophia Kenrick. Robin Curtis also thanks Jai Pathak and Prasad Sarangapani (at MedImmune) for relevant scientific discussions. mAb1 was kindly supplied by MedImmune.

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