Specific Buffer Effects on the Intermolecular Interactions Among Protein Molecules at Physiological pH

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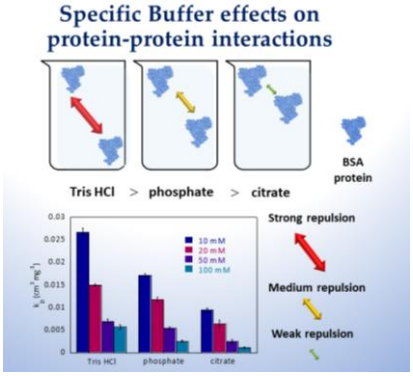
ABSTRACT. BSA and lysozyme molecular motion at pH 7.15 is buffer specific. Adsorption of buffer ions on protein surfaces modulates the protein surface charge and thus protein-protein interactions. Interactions were estimated by means of the interaction parameter k_D obtained from plots of diffusion coefficients at different protein concentrations (**$D_{app} = D_0 [1+k_D C_{protein}]$**) via dynamic light scattering and nuclear magnetic resonance. The obtained results agree with recent findings confirming doubts on the validity of the Henderson-Hasselbalch equation, which has traditionally provided a basis for understanding pH buffers of primary importance in solution chemistry, electrochemistry and biochemistry.

TOC GRAPHICS



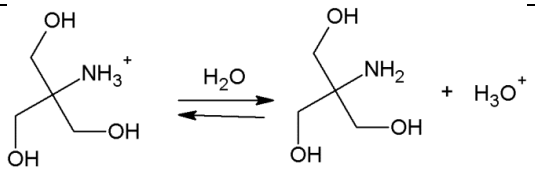
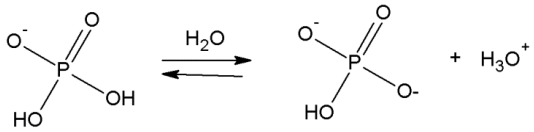
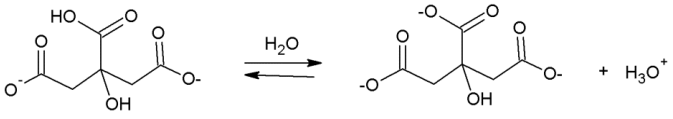
KEYWORDS: Hofmeister series, Specific buffer effects, BSA protein, interaction parameter, electrostatic interaction.

Ions play particular key roles in nature which have only been partially understood.[1–7] Specific ion effects were firstly observed by Hofmeister in 1888 who studied the salt-induced aggregation of egg white proteins.[8,9] This finding led to a myriad of studies devoted to investigate and rationalize ion specificity in solution chemistry, biochemistry and colloidal science.[1,3] Ions affect specifically several physico-chemical parameters, such as viscosity[10] and surface tension[11] of aqueous solutions and other properties like solubility,[12–16] molecular motion,[17] surface charge,[18] adsorption[19] of proteins and other macromolecules. Likewise, ions also affect biological processes, including enzyme activities[20–25] and bacterial growth,[26,27] in a way which is still unexplained. Particularly interesting is that ion specificity plays a key role at concentrations of 0.1-0.15 M typical of living systems.[24,28] At these concentrations electrostatics is screened enough to be comparable with, usually neglected, ionic van der Waals forces.[29,30] Thus, biology operates in conditions where ion specificity modulates biomacromolecules interactions.

Another effect often ignored is that due to pH buffers.[31] Weak acids/bases and their conjugate bases/acids in aqueous solutions are known to act as pH buffers. Buffers are used in chemistry and biochemistry to set pH. In living systems, buffers set pH of biological fluids which in turn regulate the protonation state of ionizable groups of biomacromolecules and thus their interactions or their biological activities.[32–34] Textbooks explain buffer action by mean of the Henderson-Hasselbalch equation: $pH=pK_a+\log [Salt]/[Acid]$.[35] It requires the knowledge of the weak electrolyte's pK_a, since buffer action can only occur within a range of $pH = pK_a \pm 1$. What is not said, but implicitly considered, is that ideally any acid/conjugated base pair (with a suitable pK_a) can be used to obtain the desired pH, irrespective of the chemical nature of the electrolyte used to set pH. That is, conventional application of the Henderson-Hasselbalch equation assumes

the system is indifferent to the specific identity of the buffer. However, some recent experiments show that, even at the same nominal pH, the chemical nature of the buffer plays a role that cannot be ignored. This is particularly true for proteins and other biointerfaces.[31] Kim et al. reported the first pioneering work on the specific effect of buffers measuring the activity of restriction enzymes.[36] After that, buffer specificity was observed for lipase activity,[37] lysozyme electrophoretic mobility,[38] antibody aggregation,[32,39] lysozyme adsorption on ordered mesoporous silica,[40] binding of a cationic dye to heparin,[41] amyloid fibril formation mechanism,[42] and other works.[43–46] Differently from the specific effects of strong electrolytes, for which a myriad of experiments and different theoretical approaches are available,[47–51] for buffers there is much less experimental evidence and, moreover, no theory has been developed yet.

Table 1. Buffers used in this work and their pK_a values at 25°C. Extracted from Ref.[52]

Buffer name	**Acid/base equilibrium**	**pK_a**
Tris [Tris(hydroxymethyl) aminomethane]		8.06
Phosphate		7.22
Citrate		6.40

Here, the specific effect of buffers to modulate protein-protein interactions was investigated by measuring the apparent diffusion coefficient, D_{app}, of BSA and lysozyme proteins through dynamic light scattering (DLS) and nuclear magnetic resonance (NMR). Three different pH

buffers - namely, Tris-HCl, sodium phosphate and sodium citrate (Table 1) - at the same nominal pH (7.15) as a function of protein concentration ($C_{protein}$) were used. Experimental DLS data followed the relationship,[53]

$$D_{app} = D_0 (1+k_D C_{protein}) \quad (1)$$

Where, D_0 is the diffusion coefficient when the protein concentration $C_{protein} \rightarrow 0$; k_D is an interaction parameter,[53]

$$k_D = 2MB_{22} - k_f - \overline{\nu} \quad (2)$$

where, M is the molecular mass of the protein, k_f is a hydrodynamic friction virial coefficient, $\overline{\nu}$ is the specific volume of the protein, and B_{22} is the osmotic second virial coefficient,

$$B_{22} = 2\pi \int_0^\infty \left[1 - exp\left(\frac{-U_r}{k_B T}\right)\right] r^2 dr \quad (3)$$

Where, U_r is the interparticle interaction potential.[54] The sign of k_D has the same meaning of that of B_{22}, that is protein-protein repulsion for k_D (B_{22}) > 0, attraction for k_D (B_{22}) < 0, and no interaction for k_D (B_{22}) = 0. Eq. (1) represents a straight line with slope m = D_0 k_D. At the molecular level, protein-protein repulsion/attraction is mainly due to adsorption of counterions driven by protein charge (Z_p),[55]

$$Z_p = \sum_i \frac{N_i}{1+10^{-pK_{ai}+pH+e\phi(r)/kTln10}} - \sum_j \frac{N_j}{1+10^{pK_{aj}-pH-e\phi(r)/kTln10}} \quad (4)$$

where N_i and N_j are the number of basic and acidic amino acid residues having the dissociation constants pK_{ai} and pK_{aj} respectively, e is the elementary charge, ϕ(r) is the surface potential, k is the Boltzmann constant and T the absolute temperature. Within this approach, Z_p depends on the surface pH_s (=pH-$e\phi(r)/k_BT$ln10), which in turn depends on bulk pH whatever the buffer used to set it. Z_p is zero (no repulsion) at pH = pI (isoelectric point) and is ≠ 0 for pH ≠ pI. If we consider a protein solution as a colloidal system[54] DLVO theory would predict an attraction for pH = pI due to van der Waals forces and a repulsion at pH ≠ pI due to the presence of the counterion

adsorption layer. Hence, whatever the buffer used to set pH, the same repulsive or attractive interaction should be obtained. But this is not so.

Figure 1 shows the specific effect of buffers on the D_{app} *vs* $C_{protein}$ plot for lysozyme (Figure 1A) and BSA (Figure 1B) proteins for different 10 mM buffers at the same nominal pH = 7.15 (298 K). A D_0 value of about 13×10^{-11} m^2 s^{-1} for lysozyme and about 5.7×10^{-11} m^2 s^{-1} for BSA were obtained. They correspond to a hydrodynamic radius, R_H, of 1.84 nm and 3.8 nm for lysozyme and BSA, respectively. These R_H values, calculated by the Stokes-Einstein relationship ($D_0 = k_BT/6\pi\eta R_H$), agree with the expected values.[56,57]

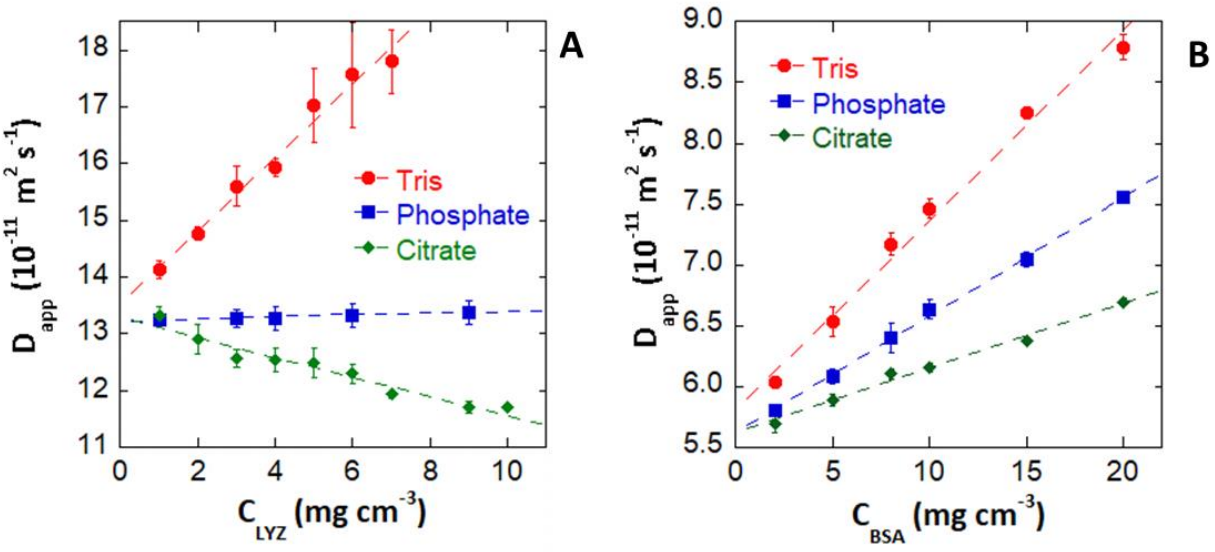


Figure 1. Specific buffer effects on diffusion coefficients of (A) lysozyme vs (B) BSA, as a function of protein concentration at 298 K. pH maintained by 10 mM buffers at 7.15.

Both plots show that the slopes of the lines, and hence k_D values, are buffer specific. For lysozyme, $k_D > 0$ (+0.048 cm^3 mg^{-1}) for Tris-HCl, $k_D \approx 0$ for phosphate (+0.0013 cm^3 mg^{-1}) and $k_D < 0$ for citrate (-0.013 cm^3 mg^{-1}). This trend agrees with that found for the electrophoretic mobility of lysozyme at pH 7.15.[38] For BSA, by contrast, k_D was positive for all buffers but with a decreasing slope along the series Tris-HCl > sodium phosphate > sodium citrate. Lysozyme has an isoelectric point pI ≈ 11, and thus carries a positive net charge at pH 7.15. It is hence likely

that the different k_D values are due to a specific adsorption (chemisorption) of chloride and anionic buffer species on lysozyme surface which affects the effective surface charge and, thus, the interaction between protein molecules. If so, chloride (the counter ion of Tris buffer) is adsorbed to lysozyme surface at a lower extent than phosphate and citrate ions. In particular, we note that k_D is almost zero for sodium phosphate and even negative for sodium citrate. This means that lysozyme molecules pass from repulsion to attraction by changing the type of buffer at the same nominal pH. This buffer specific result is remarkable and important for biochemical experiments. It is also consistent with what previously observed for lysozyme electrophoretic mobility[38] and adsorption on mesoporous silica.[40] Even more interesting is the result shown in Figure 1B. BSA has a pI ca. 4.7[57] and so negatively charged at pH 7.15. Hence, a stronger effect of cations rather than anions is expected. The trend of k_D values for BSA could be explained by a lower adsorption of $TrisH^+$ than Na^+ (the counterion of both phosphate and citrate). Nonetheless, a specific co-ion effect (phosphate and citrate) is at work since k_D is lower for the ion pair sodium citrate than sodium phosphate. The buffer specificity for BSA could be due to a direct effect of cations mediated for phosphate and citrate buffers through the different interactions of these anions with sodium cations. Alternatively, a specific interaction of negatively charged anions (chloride, phosphate, citrate), on the negatively charged BSA surface might be at work. This might be possible considering that although BSA is negatively charged at pH 7, it still has some localized positive charges which might act as anion binding sites. This effect, together with the direct cation binding, results in a buffer specific interaction parameter k_D decreasing along the series Tris-HCl > sodium phosphate > sodium citrate. In summary, at buffer concentration 10 mM and pH 7.15 the intermolecular interactions of both lysozyme and BSA proteins are buffer specific with k_D values decreasing in the same qualitative order. That is quite surprising since the

net charge of the two proteins are opposite at pH 7.15. This fact was found to be the reason of the inversion of the Hofmeister series in other cases.[14]

This suggestion is supported by NMR self-diffusion and relaxation data.[58] The NMR self-diffusion coefficient[59] of BSA in the three 10 mM buffers is in the range 4.6-5.0 × 10^{-11} m^2 s^{-1} (Table 2). Considering that the diffusion coefficient of $TrisH^+$ cation in the absence and in the presence of BSA decreases very slightly from 4.7 to 4.6 × 10^{-10} m^2 s^{-1}, a molar fraction $x_b \approx 0.03$ of bound cation to BSA is calculated from the relationship,[60]

$$D_{obs(buffer)} = x_b D_{BSA} + (1 - x_b) D_{free(buffer)} \qquad (5)$$

where $D_{obs(buffer)}$, D_{BSA}, and $D_{free(buffer)}$ are the self-diffusion coefficients observed for the buffer in the presence of BSA, for BSA protein in buffer solution, and for free buffer, respectively. The ^{35}Cl NMR signal of the Cl^- anion can be detected in the free buffer, but disappears in the presence of BSA thus indicating a relatively significant binding to BSA (^{35}Cl is a quadrupolar nucleus characterized by low sensitivity in the presence of an asymmetric environment).[61] In the case of citrate anion, $D_{free(buffer)}$ is 4.1 × 10^{-10} m^2 s^{-1} but in the presence of BSA two different self-diffusion coefficients are determined, namely 3.78 and 1.54 × 10^{-10} m^2 s^{-1}, (values (a) and (b) for D_{obs}(Buffer-BSA) in Table 2) from the bi-exponential decay of the Pulse Gradient Spin Echo (PGSE) intensity measured by NMR as a function of gradient strength (Figure S1 in Supporting Information). This finding clearly indicates that citrate anion can bind to BSA interface via two different interaction sites with different strengths, that is, different binding constants. Applying Equation 5, from the two values of D_{obs}(buffer-BSA) – the (a) and (b) values in Table 2 – we can calculate the molar fraction of bound citrate $x_b \approx 0.08$ and 0.7, respectively. This suggests that the multivalent citrate anion is strongly bound at least to one BSA site, likely characterized by nearby positive charges, via electrostatic interactions.

Table 2. ^{1}H NMR self-diffusion coefficients of BSA in 10 mM buffers prepared in D_2O at 298 K.

Buffer	**D_{free} (buffer)** ($\times 10^{-10}$ m^2 s^{-1})	**D_{obs} (Buffer-BSA)** ($\times 10^{-10}$ m^2 s^{-1})	**D_{BSA} in buffers** ($\times 10^{-11}$ m^2 s^{-1})	**x_b**
Tris-HCl	4.73±0.01	4.61±0.02	4.55±0.05	0.027
Sodium citrate[a]	4.10±0.01	(a) 3.78±0.05; (b) 1.54±0.06	4.66±0.07	(a) 0.088; (b) 0.7
Sodium phosphate	-	-	5.01±0.06	-

[a] Sodium citrate buffer: (a) and (b) D_{obs} values are obtained from the best fitting of the experimental data reported in Figure S1, whereas (a) and (b) x_b values are the corresponding binding molar fraction calculated through Eq. 5 for citrate anion in the presence of BSA.

^{23}Na and ^{31}P NMR relaxation measurements[62–64] demonstrate that Na^+ cation is more strongly bound to BSA in the case of citrate buffer, compared against phosphate buffer (Table 3). Unfortunately, since no information on relaxation rates for bound buffer ions are available, we cannot quantify their bound molar fraction, using an equation like that used for self-diffusion measurements (Eq. 5). Only qualitative information can be deduced considering that bound ions are expected to display higher relaxation rates than hydrated free ions as a result of the binding and the vicinity of many other nuclei.[62] Indeed ^{23}Na NMR spin-lattice (R_1) and spin-spin (R_2) relaxation rates $R_1 = R_2 = 22$ s^{-1} are measured in both citrate and phosphate buffers, whereas in the presence of BSA these values slightly increase in the case of phosphate ($R_1 = 23$, $R_2 = 26$ s^{-1}) and almost double in the case of citrate buffer ($R_1 = 38$, $R_2 = 41$ s^{-1}). The increase of relaxation rate is clearly related to the degree of binding. ^{31}P NMR spin-lattice and spin-spin relaxation rates are $R_1 = 0.104$ s^{-1}, and $R_2 = 3.28$ s^{-1} for free phosphate and $R_1 = 0.214$ s^{-1} and $R_2 = 18.18$ s^{-1} (see note "a" in Table 3) in the presence of BSA. Besides the possible contribution due to chemical shift anisotropy that may affect R_2 values, these data indicate a significant binding of phosphate anion to BSA sites.

Table 3. ^{23}Na and ^{31}P NMR relaxation rates of 10 mM sodium citrate and sodium phosphate buffers in the absence and in the presence of BSA (R_1 and R_2 values in s^{-1}) at 298 K.

Buffer	R_1 (free)	R_2 (free)	R_1 (with BSA)	R_2 (with BSA)
Sodium citrate (^{23}Na)	22.5±0.4	22.5±0.4	37.9±0.5	41.3±0.7
Sodium phosphate (^{23}Na)	21.8±0.3	21.8±0.3	23.1±0.5	26.2±0.5
Sodium phosphate (^{31}P)[a]	0.104±0.002	3.28±0.05	0.214±0.006	18.2±0.1

[a] ^{31}P NMR relaxation in homogeneous systems is expected to be mainly determined by dipolar relaxation mechanism. In these systems the shape of ^{31}P NMR signal is not Lorentzian thus indicating a chemical shift anisotropy contribution particularly effective to spin-spin relaxation rates.[65]

Remarkably, and in agreement with DLS results, NMR data clearly suggest that in terms of binding to BSA, cations follow the decreasing sequence Na^+ (citrate) > Na^+ (phosphate) > $TrisH^+$, whereas anions follow the decreasing sequence citrate > phosphate ≥ chloride.

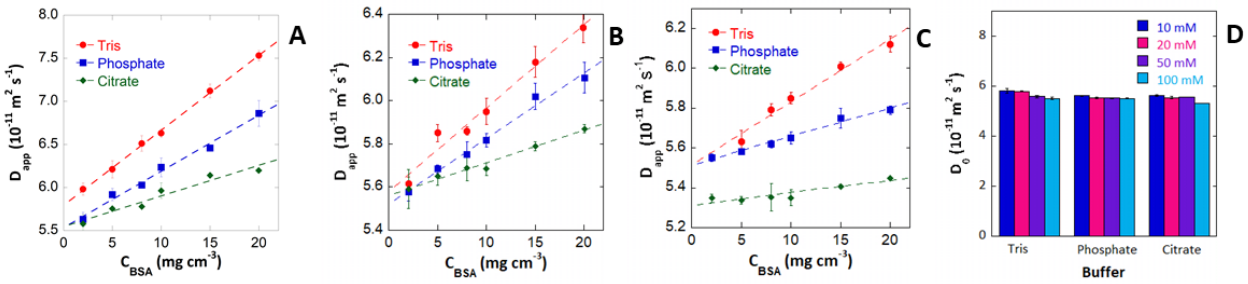


Figure 2. Effect of buffer type and concentration on the diffusion coefficient of BSA with buffer concentration (A) 20 mM, (B) 50 mM, (C) 100 mM, as a function of BSA concentration C_{BSA}. (D) Ideal diffusion coefficient, D_0, in the dilute protein limit. T= 298 K and pH maintained by (20, 50, and 100 mM) buffers at 7.15.

Figure 2 shows the effect of buffer concentration on the D_{app} *vs* BSA concentration plots. The increase of buffer concentration has the effect of decreasing the slope of the straight lines. It is also observed that the intercept of the lines is about the same for all buffers at all concentrations meaning that the D_0 (5.6×10^{-11} ± 0.2 m^2 s^{-1}), and hence the hydrodynamic radius calculable by Stokes-Einstein relationship, is unaffected by buffer type. The decrease of k_D with the increase of buffer concentration are interpreted as a decrease of repulsive interactions among protein molecules due to surface charge screening. Although this effect is general for all buffers, each buffer behaved specifically as depicted in Figure 3A.

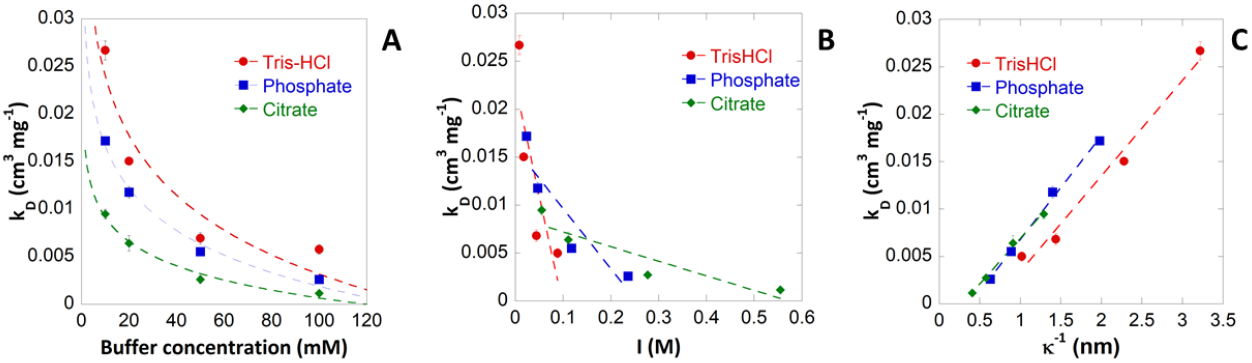


Figure 3. Dependence of k_D on (A) buffer concentration (the dashed lines are guides for the eyes); (B) ionic strength; (C) on Debye length (κ^{-1}) at 298 K and pH = 7.15.

Although buffers have same concentrations their different behavior might be due the different ionic strengths due to the different charges carried by buffer ions. Recent developments have shown how electrostatic and dispersion forces cooperate to give ion specificity.[48] Within this approach concentration profiles and electrostatic potentials are calculated using a modified Poisson–Boltzmann model, with Boltzmann concentration profiles including nonelectrostatic ion interactions $U_i^{NES}(z)$ alongside the electrostatic $\psi(z)$,

$$c_i = c_{i,0} exp\left[-\left(q_i\psi(z) + U_i^{NES}\right)/kT\right] \qquad (6)$$

The nonelectrostatic ion interactions are predominantly represented by ionic dispersion potentials.[66] The dispersion interaction is determined by the dynamic polarizability at optical/UV frequencies, modulated by the dielectric spectrum of the solvent and the surface. These parameters have not been calculated yet for buffer ions. Hence, in first approximation we consider a traditional approach based on electrostatics only looking at correlations with ionic strength or other related electrostatic parameters. Figures 3B and 3C show k_D as a function of ionic strength (I) and Debye length (κ^{-1}), respectively. We recall that κ^{-1}, which is a function of

the ionic strength ($\kappa^{-1} \propto 1/\sqrt{I}$), estimates the screening of surface potential due to the charge and concentration of the adsorbed ions. There is no direct linear correlation between k_D and I (Figure 3B, and Table S1 in supporting information), rather, k_D correlates well with κ^{-1} (i.e. $I^{-1/2}$) (Figure 3C, Table S1), particularly for phosphate and citrate buffers. We interpret this observation considering the equilibria of buffer species (Table 1). Indeed, phosphate buffer is due to the equilibrium between a monovalent/divalent ion while for citrate divalent/trivalent ions occur. For TrisHCl buffer, instead, a neutral molecule is in equilibrium with a monovalent ion. It is hence clear that electrostatic screening is more important for highly charged species than for neutral or monovalent ions. The lowest correlation coefficient with the screening length κ^{-1} is indeed observed for Tris-HCl buffer. It is likely that for this buffer non-electrostatic forces are responsible for the observed deviation from linearity.

In summary, we have observed that the molecular motion of BSA protein at physiological pH is buffer specific. The parameter k_D, which is a due to intermolecular interactions, is buffer type and concentration dependent. The repulsion among BSA protein molecules decreased as buffer concentration and ion charges were increased. Tris-HCl buffer resulted in the highest repulsion among BSA proteins, likely because Tris-H^+ ion interacts less than Na^+ with the negative groups of BSA. NMR measurements carried out for BSA in 10 mM buffer solutions in D_2O at pH 7.15 confirm the specific binding of buffer ions to the protein surface. Additionally, k_D is buffer specific in a range of salt concentration (10-100 mM) which is relevant for living organisms. The recent theory based on the implementation of Ninham's ion dispersion forces[67] effect of ions will need to be extended to include buffer specific effects. Future work will be devoted to exploring the effect of other anionic buffer counterions (different by sodium).

Moreover, additional experimental and theoretical efforts will be needed to explore the effect of dissolved atmospheric gas on colloid particles as well as biomacromolecules interactions.[68]

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Supporting Information. The Supporting Information is available free of charge at https://pubs.acs.org/. DLS and NMR experimental methods and additional data.

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