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Changes in rotational diffusion as a function of protein size and crowding conditions

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Abstract

It has long been appreciated that the intracellular environment is highly crowded and a variety of approaches have been used to attempt to mimic this environment for in vitro studies. Recent studies have demonstrated that non-specific interactions between proteins play an important role in a variety of processes including rates of folding, protein stability and protein mobility and non-specific protein-protein interactions are an important consideration in any studies involving macromolecular crowding. Previous diffusion studies have focused on small (< 10 kDa) proteins and there is limited data on diffusion of larger proteins. This study extends previous work by using fluorescence anisotropy to measure rotational diffusion of 8 different proteins in the presence of a variety of macromolecular crowding agents. These studies demonstrate that non-specific interactions with bulk proteins impact rotational diffusion as a function of size and that the chemical nature of the bulk protein is more significant than the size of the bulk protein. Our work also shows that viscosity has very little impact on the rotational diffusion of each protein. While there is still much to learn about the chemical nature of the intracellular environment, this work demonstrates that the nature of the protein of interest and the environment surrounding the protein can significantly impact its rotational mobility.

Keywords

Anisotropy; Protein crowding; Molecular motion; Fluorescence, Non-specific interactions, Macromolecular crowding agents.

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INTRODUCTION

The majority of biological chemistry experimentation has been conducted in dilute solution conditions (macromolecular concentrations < 10g/L)[1]. This is in contrast to the fact that 20-40% of the cytoplasmic volume is composed of proteins, nucleic acids and other macromolecules at concentrations up to 300 g/L[2-6]. The importance of "macromolecular crowding," a term first used by Minton and Wilf in 1981,[7] has been rediscovered and a number of recent papers have highlighted important aspects of macromolecular crowding on protein stability, protein function and protein dynamics[5,8-11]. Several types of solutions have been used to mimic the cellular environment, ranging from increasing solution viscosity with glycerol to high concentrations of a variety of proteins and synthetic polymers to reconstituted cytoplasm[12-16]. Each approach simulates different properties of the cellular environment and it has become clear that nonspecific interactions between macromolecules is an important consideration in interpreting cellular data.

Rotational diffusion is typically quantified using the Stokes-Einstein-DeBye relationship (SED), $D_r = \kappa T / 8\pi\eta r^3$, while

translational diffusion is calculated by the Stokes-Einstein relationship (SE), $D_t = \kappa T / 6\pi\eta r$, where η is viscosity, κ is the Boltzman constant and r is the protein's radius[17-19]. The SED relationship assumes that the protein is a sphere in a homogeneous solution where the test protein is much larger than the solute that is controlling the viscosity[20]. However, this is typically not the case since many proteins are not spherical and the macromolecular distribution within the cytoplasm is very heterogeneous.

Recent studies on protein diffusion have shown that non-specific interactions between proteins play an important role in protein mobility, resulting in deviations from both the SED and SE relationships[10,16,21]. NMR diffusion studies using chymotrypsin inhibitor 2 (CI2) have shown that translational and rotational diffusion decreases with increasing concentrations of synthetic polymers and greater impacts on diffusion are observed for translational diffusion rather than rotational diffusion[16]. In some cases, such as for glycerol, the changes in diffusion are in accordance with Stokes-Einstein and Stokes-Einstein-DeBye laws while other polymers (such as polyvinyl pyrrolidone (PVP)) deviate from predicted diffusion changes suggesting that more than viscosity changes are impacting diffusion[10]. Bulk protein macromolecular crowding agents exhibit the opposite effects of synthetic polymers with greater impacts observed on rotational diffusion rather than on translational diffusion[16]. Relaxation data demonstrated that this difference is due to nonspecific interactions between the bulk proteins and the protein of interest, which is not observed with synthetic polymers[16]. These results are supported by Brownian dynamics simulations which have shown that diffusion of macromolecules are sensitive to nonspecific attractive interactions and that diffusion of larger macromolecules can be significantly slowed down[8]. Taken together, these results highlight the importance of understanding potential interactions between the target protein and any macromolecular crowding agents.

Recent protein diffusion studies have primarily utilized NMR to measure rotational and translational diffusion and therefore have used small proteins (< 10 kDa) and there limited studies focused on the impact of macromolecular crowding on larger proteins. Fluorescence anisotropy offers an alternative to NMR to measure differences in rotational diffusion as well as providing information about shape, dimensions and flexibility of biomolecules[22-24]. In this study, we measure the anisotropy of 8 different proteins of varying molecular weight in the presence of varying amounts of 7 different crowding agents. Our results demonstrate that rotational motion is decreased as protein size increases and, consistent with other studies, non-specific interactions between the target protein and bulk protein significantly decrease rotational motion while synthetic polymers and other non-protein crowding agents exhibit less impact on rotation. Our results also suggest that the electrostatic nature of the bulk protein is more important than the size of the bulk protein. These results further demonstrate the importance of considering the molecular properties of macromolecular crowding agents in interpreting experimental data.

EXPERIMENTAL METHODS

Preparation of labeled proteins

Alexa-488 labeled bovine serum albumin (BSA) and concanavalin A (ConA) was purchased from Invitrogen. Ovalbumin, conalbumin, transferrin, aldolase, ferritin and thyroglobulin from gel filtration standard kits (GE Healthcare) were labeled using a Alexa-488 labeling kit (Invitrogen). Labeled protein was repurified by gel filtration chromatography.

Anisotropy measurements

Anisotropy was measured using a SLM Aminco 8100 fluorimeter equipped with *in situ* Glan-Thompson polarizers. Labeled protein was diluted into buffer (50 mM Tris, pH 7.5) to a total volume of 1.5 mL and the fluorescence intensity was measured with the filters in both parallel and perpendicular positions. All experiments were conducted at 20 °C (293 K) using a 1 sec integration and 20 measurements or a polarization tolerance < 0.001, whichever came first. G factors were measured at the start of each measurement. Crowding agent was titrated into the cuvette in 100 μL aliquots and the anisotropy was measured after each addition. Each experiment was conducted three times and the calculated anisotropy at each point was averaged.

Viscosity measurements

The viscosity of each solution was measured at each dilution by recording the time the solution takes to pass through the marks of a thermostated Cannon-Fenske capillary viscometer. The kinematic viscosity was calculated from the viscometer constant (VWR) of 0.0018966 centiStokes per second (cST/sec). The density of each solution was measured in triplicate with a Mettler Toledo 5 place density meter. The intrinsic viscosity was calculated as the product of the density and the kinematic viscosity.

Rotational diffusion coefficient calculations

Theoretical rotational diffusion coefficient determination

Rotational diffusion coefficient (D_r) was calculated using

the formula $D_r = \kappa T/8\pi\eta r^3$. T was determined to be 293 °K, the experimentally determined viscosity (η) for each solution was used and the experimentally determined Stokes radii in the literature were used as the radii for each protein.

Experimentally determined rotational diffusion coefficient determination

The rotational diffusion coefficient was calculated using the formula $Dr = (6\theta)^{-1}$. The rotational correlation time of the fluorophore (θ) was calculated using the formula $\theta = \tau/((r_0/r)^{-1}))$. The fundamental anisotropy (r_0) was determined by plotting 1/r by T/η to create a Perrin plot and fitting the data to a straight line. The Y-intercept of this line is $1/r_0$.

RESULTS

Previous studies on the impacts of macromolecular crowding have primarily considered small proteins < 10kDa in order to utilize NMR's ability to monitor both translational and rotational diffusion. However, the majority of proteins are larger than 10 kDa and there is limited experimental data regarding the impacts of macromolecular crowding on larger proteins. In contrast to NMR, fluorescence anisotropy can be used to measure rotational diffusion of proteins and is not limited by protein size as long as the lifetime of the fluorophore is on the order of the rotational correlation time, providing a powerful tool to consider the impacts of macromolecular crowding. A total of 8 proteins were chosen to cover a range of molecular weights and properties and each protein was labeled with the fluorescent tag Alexa-488 (TABLE 1). Seven commonly used molecular crowding agents were identified from the literature and chosen for this study (TABLE 2).

TABLE 1 : Labeled proteins and their characteristics

Labeled Proteins	Monomer Molecular Weight (kDa)	Subunits	Total Protein Molecular Weight (kDa)	pI	Charge at pH 7.5	Radii (nm)
Ovalbumin	43		43	4.6	Anion	2.7[35]
Bovine Serum Albumin (BSA)	66.8		66.8	4.7	Anion	3.5[36]
Conalbumin	75		75	6.6	Neutral	4.0[37]
Transferrin	80		80	5.5	Anion	4.0[38]
Concanavalin A (ConA)	26.5	4	106	4.5-5.5	Anion	4.2[39]
Aldolase	156		156	8.6	Cation	4.8[40]
Ferritin	19-21	24	444	4.4	Anion	6.1[40]
Thyroglobulin	330	2	669	4.4-4.7	Anion	8.5[40]

TABLE 2 : Properites of crowding agents

Crowding Agents	Crowding Agent Stock Concentrations	Molecular Weight (kDa)	pI	Nature at pH 7.5
Glycerol	42% (V/V)	0.090	NA	Polar
Polyvinylpyrrolidone (PVP)	300 g/L	40	NA	Polar
Ficoll 70	200 g/L	70	NA	Polar
Sucrose	75% (W/V)	0.34	NA	Polar
Lysozyme	200 g/L	15	11.0	Cation
Ovalbumin	200 g/L	45	4.6	Anion
Bovine Serum Albumin (BSA)	200 g/L	66	4.7	Anion

NA: not applicable

The crowding agents were chosen to include both bulk proteins and non-protein based macromolecular crowding agents. Three commonly used protein based crowding agents (ovalbumin, BSA and lysozyme) were chosen for these studies[12]. Proteins have been suggested to serve as good macromolecular crowding agents since proteins can occupy up to 30% of the cell's volume at concentrations up to 400 g/L[3] and they have a much greater variety of charges and shapes than most other polymers, providing many potential possible non-specific interactions. 4 non-protein macromolecular crowding agents (Ficoll-70, PVP, sucrose and glycerol) have also commonly been used in other studies[13,15,25]. Ficoll-70, PVP and sucrose all have fairly regular charge distributions and are believed to primarily mimic crowded through volume exclusion effects[26]. The final effect we wanted to consider was increased viscosity rather than volume exclusion effects. Glycerol and sucrose have been used in previous studies[16] and they provide a measure of the impact of viscosity changes in the absence of any significant non-specific interactions as glycerol stabilizes proteins by increasing the chemical potential of the protein so that unfolding becomes unfavourable[27].

We initially compared the anisotropy of each of our test proteins in the absence of any macromolecular crowding agents to determine the impact of molecular weight on rotational diffusion in dilute solution conditions (50 mM Tris, pH 7.5). Anisotropy increases as a function of molecular weight with smaller proteins (< 100 kDa) and then fluctuates with the larger proteins used in this study (TABLE 3). However, there is only a molecular weight difference of 15 kDa between BSA and transferrin but the transferrin anisotropy is about twice the BSA anisotropy, suggesting that some of our test proteins may exhibit a higher degree self-association than other. This could also explain why ferritin's anisotropy is significantly smaller than the other larger proteins.

TABLE 3 : Average anisotropy of labeled protein

Protein	Total Protein Molecular Weight (kDa)	Average anisotropy
Ovalbumin	43	0.066 ± 0.006
BSA	66.8	0.079 ± 0.004
Conalbumin	75	0.131 ± 0.011
Transferrin	80	0.164 ± 0.016
Concanavalin A	106	0.158 ± 0.032
Aldolase	158	0.157 ± 0.032
Ferritin	444	0.107 ± 0.013
Thyroglobulin	669	0.138 ± 0.018

The anisotropy for each protein was then measured in the presence of increasing amount of each crowding agent. The viscosity of each crowding agent was also measured and a Perrin plot was created for each protein (representative data is shown in Figure 1 with the complete data set included as supplemental data). Bulk proteins provided the largest anisotropy changes for all the test proteins, with ovalbumin producing the largest differences, even at a very low volume fraction. Much less significant effects were seen with the non-protein crowding agents, with the exception of Ficoll-70, which exhibited similar effects as the protein crowding agents with conalbumin and aldolase. Both the PVP and Ficoll-70 did impact the anisotropy of thyroglobulin and ferritin as well, though not as much as was observed with the bulk protein agents.

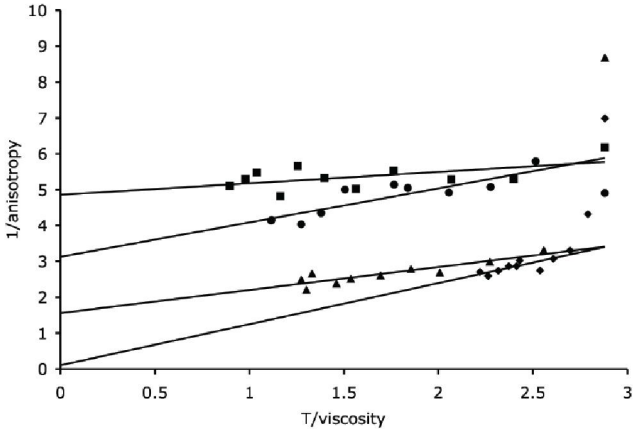

Figure 1 : Perrin plot demonstrating the change in anisotropy as a function of viscosity. Perrin plot for aldolase in the presence of lysozyme (◆), Ficoll-70 (▲), PVP (■) or sucrose (●). Lines representing the fit used to calculate 1/r0 have been included.

Interestingly, the pI of the bulk protein does not seem to be a major contributing factor. Ovalbumin as a bulk protein produced the largest changes in anisotropy while BSA and lysozyme both produced similar anisotropy increases (Figure 2). It would be anticipated that ovalbumin and BSA should have similar impacts on anisotropy and lysozyme would exhibit a different effect on the anisotropy if pI were a determining factor (TABLE 2). This is not too surprising since many proteins have a heterogeneous charge distribution and therefore have the potential to interact with both positively and negatively charged groups on other proteins.

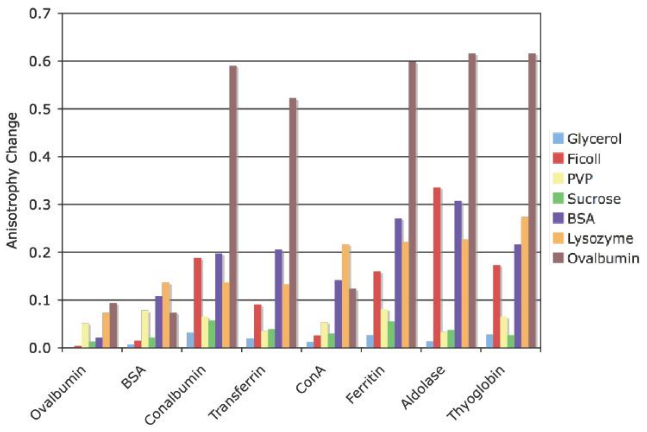

Figure 2 : Bulk protein produces the largest anisotropy changes. The measured anisotropy in the absence of any macromolecular crowding agent was subtracted from the measured anisotropy at the highest concentration of macromolecular crowding agent. As can be seen, the bulk protein crowding agents result in the largest anisotropy changes with ovalbumin producing the largest changes. Larger proteins also exhibit larger anisotropy changes.

We plotted the viscosity as a function of volume fraction for each crowding agent to determine the impact of each crowding agent on bulk solution viscosity (Figure 3). Bulk proteins do not result in significant viscosity changes (< 0.5 cP) even at high concentrations, while changes of over 2 cP were observed in some cases with the non-protein crowding agents. This clearly demonstrated that the observed anisotropy differences in the presence of bulk protein are not a function of viscosity. Results from other studies have suggested that non-specific interactions between proteins play a significant role in protein motion within the cell and these results support that conclusion[8,10,16].

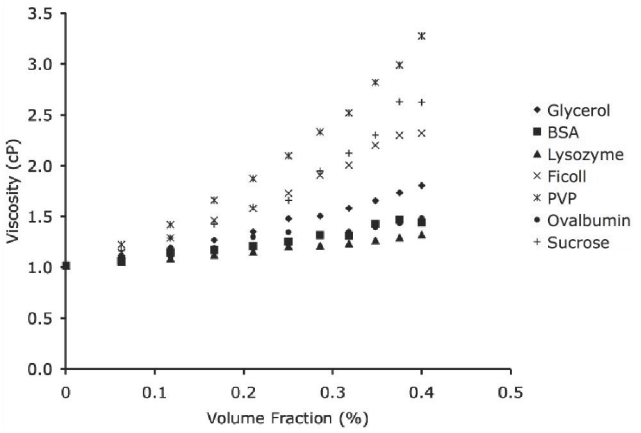

Figure 3 : Protein crowding agents exhibit small changes in viscosity while chemical crowding agents exhibit increased viscosity.

An important consideration is how each of the crowding agents impacts diffusion in relation to the predicted SED relationship. Studies with chymotrypsin inhibitor 2 (CI2) have shown that synthetic polymers produce a negative SED deviation (measued SED is smaller than the calculated SED) while bulk proteins produce a positive deviation[16]. As stated above, the SED relationship is based on the approximation that the test protein is a sphere and deviations would be predicted for non-spherical proteins. Similar deviations should be observed for the same protein in all 7 test solutions if there are no solution effects on the SED relationship. However, if solution conditions effect rotational diffusion, different deviations in the SED relationship should be observed and the same trends should be observed for most proteins.

The SED relationship was used to calculate rotational diffusions for each protein using the measured solution viscosities and Stokes radii from the literature. For each protein in each solution condition, the ratio of rotational diffusion coefficient in dilute buffer to the diffusion coefficient in crowded solution was calculated. Representative data is shown in Figure 4 with data for all proteins under all conditions provided as supplemental data. Bulk crowding agents resulted in positive deviations from the calculated rotational diffusion coefficient, while non-protein crowding agents generally result in either no deviation or a negative deviation. This suggests that, consistent with other studies, protein crowding agents impede rotation, in some cases quite severely, while our target proteins are less impacted by the non-protein macromolecular crowding agents than would be predicted. The observed effects correlate with the differences in viscosity. Bulk proteins exhibit limited viscosity differences over the range of concentrations used in these experiments while there are very large changes in viscosity for the other crowding agents. The correlation between deviations in the calculated rotational diffusion and the viscosity changes suggests that the positive deviations from the Stokes law calculations observed with bulk proteins are due to the lack of accounting for non-specific interactions.

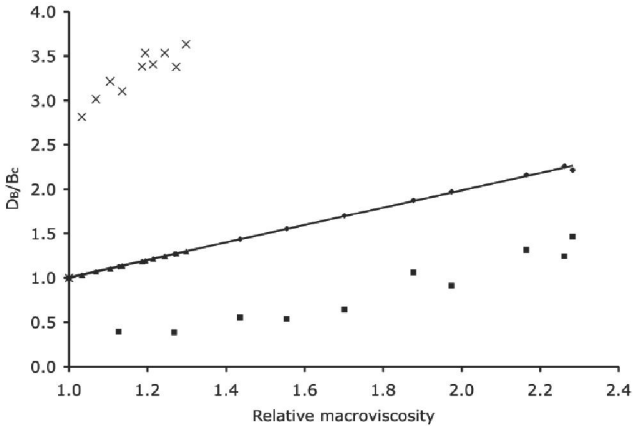

Figure 4 : Ratio of rotational diffusion coefficients in dilute buffer (DB) and in crowded solution (DC). The diffusion coefficient in dilute buffer (no crowding agent) was divided by the diffusion coefficient in a crowded solution for both the theoretically calculated (Ficoll-70 (◆) and lysozyme (▲)) and experimentally (Ficoll-70 (■) and lysozyme (x)) determined rotationally diffusion coefficients.

DISCUSSION

The cellular environment is unique in its crowded and nonhomogeneous nature and properly mimicking this environment has been a challenge. One aspect of the cellular environment that has become of particular interest is the role of non-specific and weak interactions in living cells[28]. That it is has taken this long to appreciate the role of these interactions in the cytoplasm is a little surprising since weak interactions are such a critical part of biochemistry, especially in organizing cellular networks and metabolic pathways[29,30]. Several recent studies have reported the effects of common macromolecular crowding agents on protein translational and rotational diffusion but previous studies have primarily focused on small proteins. This study adds to the existing body of work by investigating the impact of different macromolecular crowding agents on proteins with a range of molecular weights.

In analyzing the data presented here, we considered the following variables: molecular weight of the target protein, charge on the target protein, chemical nature of the molecular crowding agent and impact on solution viscosity of the crowding agents and several general principles are evident from our results. First, in general, larger proteins exhibit higher anisotropies in dilute solution conditions but there are exceptions to this rule, such as ferritin, which suggests that self-association plays a role in rotational

diffusion. As mentioned above, the low anisotropy measured with ferritin could be a function of either weak self-association or dissociation of the ferritin complex. Second, larger anisotropy changes are measured for larger proteins regardless of the nature of the crowding agent. Third, bulk protein reduces rotational diffusion much more than synthetic polymers or other macromolecular crowding agents presumably through non-specific interactions. Fourth, size of the bulk protein is not a determining factor in the degree of non-specific interactions that occur as ovalbumin produces the largest effect on rotational diffusion but it is smaller than BSA and larger than lysozyme. Finally, increasing bulk protein concentrations results in minimal differences in viscosity while synthetic polymers result in large viscosity changes. These results will help develop solution conditions that more accurately reflect the cytoplasmic environment.

One very clear principle that has arisen from this study and the other recent work in this area is that increased solution viscosity is not sufficient to model the cellular environment. On the other hand, bulk protein, while producing non-specific interactions with the target protein, may not be able to achieve sufficient viscosity to truly mimic the cellular environment though more accurate measurements of the cellular viscosity are necessary. Early experiments using ESR calculated the cytoplasmic viscosity to be between 2-3 cP depending on cell type[31], which is higher than any of the bulk protein solutions used in the study but lower than the viscocities of the polymer solutions. More recent studies using microfluorimetry have shown that the viscosity near the plasma membrane can be as low 1.0 cP[32], though it is not clear if the viscosity throughout the cystoplasm is consistent. It has also been shown that GFP diffusion is faster in eukaryotic cells than in bacteria, though it has not been conclusively shown whether is this due to a lower cytoplamic viscosity or from differences in interactions cellular compoents such as the cytoskeleton[33,34]. Based on current data, it is plausible that cellular viscosity may vary as a function of cell type, cellular compartment and/or cellular crowding but current data is not sufficient to accurately model the viscosity of the cellular environment and future studies will be necessary in this area.

We propose that the ideal solution to mimic the cellular environment would be a heterogeneous solution containing both bulk protein to create weak interactions with the protein of interest along with a synthetic polymer to increase solution viscosity. Our results also suggest that a heterogenous mixture of bulk proteins would also be ideal since different proteins interact non-specifically in different ways. BSA and lysozyme interact non-specifically with most of the proteins we tested to a similar extent but ovalbumin appears to significantly decrease rotational diffusion. It is likely the ratio of BSA and ovalbumin could be tuned to produce specific rotational diffusion effects. A systematic approach of varying solution conditions will be necessary to develop conditions with the proper viscosity and ratio of non-specific interactions. This will require a better understanding of both cellular viscosities and diffusion coefficients than currently exists. It is also likely that different cell types will exhibit different properties and solutions will need to be tuned to the cellular environment though additional data is necessary to truly assess this possibility.

One challenge with macromolecular crowding is being able to measure an effect of interest in the presence of crowding agents, especially bulk protein crowding agents, since many of the commonly used signals for monitoring protein interactions and conformational changes are masked by the bulk protein. For instance, a common technique for monitoring protein folding is intrinsic fluorescence, which primarily relies on tryptophan fluorescence. This approach becomes intractable in the presence of high concentrations of bulk protein, meaning that folding studies conducted in the presence of bulk proteins will require the development of creative new experimental approaches. As better approaches for modeling the cellular environment in the test tube are developed, concurrent method development will be important to utilize these new solution conditions.

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REFERENCES

[1] G.J.Pielak; Proc.Natl.Acad.Sci.U S A, **102**, 5901 (**2005**).
[2] R.J.Ellis; Trends Biochem.Sci., **26**, 597 (**2001**).
[3] K.Luby-Phelps; Int.Rev.Cytol., **192**, 189 (**2000**).
[4] S.R.McGuffee, A.H.Elcock; PLoS Comput.Biol., **6**, e1000694 (**2010**).
[5] J.T.Mika, B.Poolman; Curr.Opin.Biotechnol., **22**, 117 (**2011**).
[6] S.B.Zimmerman, S.O.Trach; J.Mol.Biol., **222**, 599 (**1991**).
[7] A.P.Minton, J.Wilf; Biochemistry, **20**, 4821 (**1981**).
[8] T.Ando, J.Skolnick; Proc.Natl.Acad.Sci.U S A, **107**, 18457 (**2010**).
[9] L.M.Charlton, C.O.Barnes, C.Li, J.Orans, G.B.Young, G.J.Pielak; J.Am.Chem.Soc., **130**, 6826 (**2008**).
[10] C.Li, L.M.Charlton, A.Lakkavaram, C.Seagle, G.Wang, G.B.Young, J.M.Macdonald, G.J.Pielak; J.Am.Chem.Soc., **130**, 6310 (**2008**).

[11] A.C.Miklos, M.Sarkar, Y.Wang, G.J.Pielak; J.Am.Chem.Soc., **133**, 7116 (2011).
[12] J.Li, S.Zhang, C.Wang; J.Biol.Chem., **276**, 34396 (2001).
[13] Z.M.Lin, S.Li; Protein Pept.Lett., **17**, 1426 (2010).
[14] B.van den Berg, R.J.Ellis, C.M.Dobson; EMBO.J., **18**, 6927 (1999).
[15] B.van den Berg, R.Wain, C.M.Dobson, R.J.Ellis; EMBO.J., **19**, 3870 (2000).
[16] Y.Wang, C.Li, G.J.Pielak; J.Am.Chem.Soc., **132**, 9392 (2010).
[17] P.J.W.Debye; Polar Molecules; The Chemical Catalog Company, Inc.: New York, (1929).
[18] A.Einstein; Ann.Phys-Berlin, **19**, 371 (1906).
[19] A.Einstein, R.Fürth, A.D.Cowper; Investigations on the Theory of the Brownian Movement; Methuen & Co. Ltd., London, (1926).
[20] C.Li, Y.Wang, G.J.Pielak; J.Phys.Chem.B., **113**, 13390 (2009).
[21] S.Zorrilla, M.A.Hink, A.J.Visser, M.P.Lillo; Biophys.Chem., **125**, 298 (2007).
[22] D.C.Yang, W.E.Gall, G.M.Edelman; J.Biol.Chem., **249**, 7018 (1974).
[23] J.Yguerabide, H.F.Epstein, L.Stryer; J.Mol.Biol., **51**, 573 (1970).
[24] J.R.Lakowicz, G.Freshwater, G.Weber; Biophys.J., **32**, 591 (1980).
[25] A.C.Miklos, C.Li, N.G.Sharaf, G.J.Pielak; Biochemistry, **49**, 6984 (2010).
[26] J.Martin; J.Mol.Recognit., **17**, 465 (2004).
[27] K.Gekko, S.N.Timasheff; Biochemistry, **20**, 4667 (1981).
[28] P.A.Srere; Trends Biochem.Sci., **25**, 150 (2000).
[29] P.Durek, D.Walther; BMC Syst.Biol., **2**, 100 (2008).
[30] E.H.McConkey; Proc.Natl.Acad.Sci.U S A, **79**, 3236 (1982).
[31] A.M.Mastro, M.A.Babich, W.D.Taylor, A.D.Keith; Proc.Natl. Acad.Sci.U S A, **81**, 3414 (1984).
[32] S.Bicknese, N.Periasamy, S.B.Shohet, A.S.Verkman; Biophys.J., **65**, 1272 (1993).
[33] R.J.Ellis; Curr.Opin.Struct.Biol., **11**, 114 (2001).
[34] M.B.Elowitz, M.G.Surette, P.E.Wolf, J.B.Stock, S.Leibler; J.Bacteriol., **181**, 197 (1999).
[35] M.A.Qasim, A.Salahuddin; J.Biochem., **85**, 1029 (1979).
[36] M.T.Ribela, P.Bartolini; Anal.Biochem., **174**, 693 (1988).
[37] J.L.Coffman, E.N.Lightfoot, T.W.Root; Journal of Physical Chemistry B, **101**, 2218 (1997).
[38] E.M.Wilson, F.S.French, P.Petrusz; Cancer Res., **42**, 243 (1982).
[39] E.Ahmad, A.Naeem, S.Javed, S.Yadav, R.H.Khan; J.Biochem., **142**, 307 (2007).
[40] M.Wyss, R.Brugger, A.Kronenberger, R.Remy, R.Fimbel, G.Oesterhelt, M.Lehmann, A.P.van Loon; Appl.Environ. Microbiol., **65**, 367 (1999).