Lysozyme Mutual Diffusion in Solutions Crowded by Poly(ethylene glycol)

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Received March 14, 2006; Revised Manuscript Received April 13, 2006

ABSTRACT: Mutual diffusion in a multicomponent system at high level of macromolecular crowding has been investigated for a solution containing a protein and a polymeric precipitant. Specifically, precise mutual diffusion coefficients for the ternary system lysozyme (component 1)−PEG2000 (component 2)−water (component 0) up to 25% in volume of PEG have been determined via Gouy interferometry. Experimental diffusion data, D_{ij}, are compared to those predicted by two semiemprirical equations, which are discussed according to the physics behind them. D_{ij} values are used to analyze the gravitational stability of the protein−polymer aqueous solution, and, in combination with the Onsager reciprocal relations, to evaluate the lysozyme salting out coefficient for addition of PEG (μ_{12}). Experimental μ_{12} are in good agreement with those predicted by Lekkerkerker's equation. All the results have been compared to those corresponding, previously determined, for a companion system containing a PEG sample at a different molecular weight, PEG400. The influence of PEG-induced crowding on proteins in physiological or in crystallization media has been discussed.

I. Introduction

The finite size of biomacromolecules in physiological media has many relevant implications on biological events. In fact, high concentration of "background" macromolecules induces changes in the rates and equilibria of numerous biochemical reactions.[1]

The ability of testing macromolecular crowding and macromolecular confinement in vitro requires the use of some crowding biocompatible additive. Indeed, neutral polymers, such as poly(ethylene glycol) (PEG) or dextran, are used in a wide range of biotechnological applications, ranging from purification methods to many cell-fusion applications.

Mutual and intra diffusion are properties strongly related to the free volume fraction. Therefore, they have been extensively investigated, in vivo[2,3] and in vitro,[4] in biological compartments, where a macromolecular crowding exists. As far the intradiffusion is the most extensively investigated transport property.[5]

The macromulecular crowding effect is also used in obtaining protein crystals for X-ray analysis. Three-dimensional structure of biomacromolecules is a mandatory step in the modern proteomics, and X-ray crystallography from single crystal is the only way to determine the structure of biomacromolecules with molecular weight higher than 30 kDa. In biocrystallography, the limiting factor is still the production of protein crystals with good crystallographic quality, as elsewhere reviewed.[6] Protein crystallization is also the less understood step, and the one with the worst success rate of the entire process from protein expression to its three-dimensional structure determination.[7] Many high-throughput programs are running nowadays for improving the crystallization efficiency in genomic or proteomic project.[8] Nevertheless, the crystal production is still commonly obtained via trial-and-error procedures in which the protein is slowly precipitated from a solution, adding some precipitant, as PEG (200−20 000 Da) or some salts. Herein we focus on the role of PEG on protein crystal growth.

The process of formation of a crystal can be split into two consecutive steps: (1) generation of a nucleus and (2) growth of the crystal. The first step is reached by holding the solution under strongly supersaturated conditions, and then the crystal growth process begins. Then the last step occurs, reducing the supersaturation to a lower level, and thus the growth of few large crystals is obtained. Moreover the crystal growth would have to proceed very slowly to maximize the degree of order in the crystal lattice.

The crystal growth process is essentially regulated by two parameters: the mass transport of solutes (protein, precipitants, additives, buffer) toward or out of the crystal-solution interface and the interface kinetics. The nature of mass transport is a strongly determining factor to the crystallographic quality. To obtain good quality crystals, the transport of the solutes must be limited essentially to a diffusive motion. In fact, the phase separation induces concentration gradients, responsible for diffusive transport and density gradient, generating a convective transport. The last one determines a less extensive and more impure crystal formation. Thus, the protein crystal quality is generally better if convection phenomena are depleted, as in reduced gravity environments (μg) or in gel or in very viscous media.[9]

In the perspective to optimize mass transport in crystallization reactors, the study of the diffusion appears a fundamental step to describing the mechanism of protein crystal formation. A correlation analysis between success rate in microgravity crystallization and protein diffusion properties has been recently published,[10] but an analysis on the effect of cross-diffusion of massive amounts of precipitants is still lacking in the literature. Only recently, first multicomponent diffusion data[11−15] and its applications to numerical simulations of protein crystal growth[16] have been presented to the scientific community.

Clearly, a multicomponent approach turns out mandatory for such a kind of diffusion analysis. A complete diffusion matrix

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10.1021/ma0605705 CCC: $33.50
Published on Web 06/01/2006

is required, as defined by the Ficks' law:

$$J_i = -\sum_{j=1}^{n-1} D_{ij} \nabla c_j \quad i = 1, ..., n-1 \tag{1}$$

Equation 1 describes the diffusion flow, J_i, relative of the "*i*th" solute, in a *n*-components solution. It contains the main-term diffusion coefficients, D_{ii}, which account for the flux of each component induced by its own concentration gradient, ∇c_i, and it also contains the off-diagonal cross terms D_{ij}, which account for the flux of each component on the gradient of the other component ∇c_i. Solutions from which protein crystals grow are systems in which significant cross-flows occur, essentially because of two factors: the likely insurgence of high concentration gradients at the interface between solution and growing crystal and the strong dragging cross-effect, due to protein−precipitant interactions (electrostatic forces in the case of a salt, or depletion forces in the case of PEG). Moreover, D_{ij} and partial molar volumes allow the determination of the rare and important variable μ_{12},[12,14,15] useful to understand the role of macromolecular crowding in vivo.

The present paper provides multicomponent diffusion data for protein-PEG solutions with a 2-fold scope: to test current theories about mutual diffusion in crowded solutions as a function of the free volume fraction and of the crowding agent molecular size and to shed light on the hydrodynamic and thermodynamic role of PEG in protein crystallization.

II. Experimental Section

Materials. Poly(ethylene glycol) with numerical average molecular weight 2034 Da (PEG2000) was purchased from Aldrich and used without further purification. For Aldrich PEG samples, the ratio between the weighted and numerical molecular weight is typically about $M_w/M_n = 1.06$ indicating a mild polydispersity. Hen egg white Lysozyme (14023 Da), six times recrystallized and lyophilized (lot E96302), was purchased from Seikagaku. Rosenberger and co-workers suggested this choice because of its high purity.[17] The counterion is the chloride (2.27%) with a moisture content of 3.93 wt %. No impurity effect on the interferometric data was detected in previous works,[11−14] where the same supplier was used.

Solution Preparation. The solutions have been prepared by weight, with double distilled water, on the same day of the diffusion experiments, withdrawing solutes under dry conditions, as described earlier.[14] Small difference in the PEG concentration, between the top and bottom solution for the diffusion experiments, can cause a small difference of pH and then the presence of an undesirable hydrogenionic concentration gradient across the diffusion boundary. This could be avoided using a buffer, but in order to limit the number of independent components in solutions, we preferred to adjust the pH of the diffusing solutions to a constant value (pH 4.5). For this reason, a suitable amount of hydrochloric acid was added to each counter-diffusing solution. In contrast to systems of lysozyme−salt−water,[11−13] the protein−PEG−water systems have a low ionic strength; therefore, a combined electrode with a good performance under these conditions has been used (Radiometer pHM220). The pH-meter is a DeMori pM520. The pH-meter was calibrated with the IUPAC standard buffer solutions each day that the diffusion measurements were performed. No standardization of the pH has been presented in the literature for the "mixed solvent" water-PEG2000.

Density Measurements. Density measurements were performed for all solutions prepared for diffusion experiments to convert the mass concentration to molarity. The density, *d*, has been measured at 25.00 ± 0.01 °C using an Anton PAAR densimeter, model 602. The instrument was calibrated with double distilled water and with air whose density was based on the ambient humidity and pressure, where the humidity was corrected to 25.00 °C. The following equation was fitted to the experimental data

$$d = \bar{d} + \sum_{i=1}^{n-1} H_i (c_i - \bar{c}_i) \tag{2}$$

where H_i is defined as

$$H_i = \left(\frac{\partial d}{\partial c_i}\right)_{c_j, \bar{c}_i} \tag{3}$$

and $\bar{d}$ is the density at the average concentration. The volumetric results are reported in Table 1. Also reported in Table 1 are the partial molar volume evaluated as recommended in ref 18.

Mutual Diffusion Measurements. The four D_{ij}s have been obtained by Gouy interferometry at different compositions, keeping the protein concentration constant at 0.6 mM but the PEG2000 concentration ranging from 0.025 up to 0.125 M at 25.00 ± 0.02 °C and at pH = 4.5. For each pair of solutions used in the diffusion experiments, the difference of pH was very small and lower than about 0.05 pH unit. Because of the small pH dependence of the diffusion coefficients found in a previous work,[11] it is reasonable to presume that even a difference of a tenth of a pH unit allows reasonable comparison of data at the different compositions in this system too. In each free diffusion experiment, the top and bottom solutions, for different compositions of at least one solute are layered using the siphoning technique. The large viscosity increase[19] due to solute 2 (PEG in our system) concentration makes the pulling of the boundary a more and more delicate effort. We temporarily stop pulling the boundary during the sharpening period in order to get good initial step-function-like conditions. The Gouy interferometer at the University of Naples uses a He−Ne laser source (λ = 632.8 nm), a single channel cell (a = 2.5000 cm), and the Gouy minima were recorded with a photodiode on line. When the light goes through the cell, the presence of the concentration (refractive index) gradient produces a deviation of light beams, and so an interference pattern evolving with time can be observed. A set of four measurements at the same mean concentration but at different values of ratio $\Delta c_i/\Delta c_j$ were performed, where the Δc_i is the difference in concentration of component *i* between the top and bottom solutions. Twenty scans of these fringes pattern are usually recorded at different times for each experiment. The experimental data are the positions of the fringes (50 on average) at each of the 20 scans. From these data, some literature programs are used to obtain the three Gouy parameters: J_m, the total number of Gouy interference fringes; D_A, apparent diffusion coefficient; and Q_0; the "area under the deviation function".[20] From the set of four experiments collected at different ratio $\Delta c_i/\Delta c_j$, the four D_{ij} can be determined by the Fujita−Gosting procedure,[20] with data reported in Table 1. The presence of a polymer with a mild polydispersity (Poisson distributions) makes the errors from the analysis of the interferometric data slightly higher than usual. The effects of the polydispersity on the diffusion of several polymers, included PEG, was elsewhere discussed by the authors.[21,22] The high errors associated with the D_{ij} data at c_2 = 0.05 M are due to the very similar values of the eigenvalues of the diffusion matrix. The Gouy interferometry analysis is based on the hypothesis of no volume change on mixing associated with the diffusive flow, i.e.

$$\sum_{i}^{n-1} J_i \bar{V}_i = 0 \tag{4}$$

and so it provides the D_{ij} expressed in the volume fixed reference frame, reported in Table 1.

III. Theoretical Background

There are two current theories on the effect of size mismatch between solution component, relating cross- (D_{ij}) and main- (D_{ii}) term diffusion coefficients in ternary systems. One is based on excluded volume concepts,[14,23] and the other is derived in terms

Table 1. Diffusion Coefficients and Volumetric Parameters for the System Lysozyme(1)−PEG2000(2)−Water

c_1/mol dm^{-3}	c_2/mol dm^{-3}	$D_{11}/10^{-9}$ m^2 s^{-1}	$D_{12}/10^{-9}$ m^2 s^{-1}	$D_{21}/10^{-9}$ m^2 s^{-1}	$D_{22}/10^{-9}$ m^2 s^{-1}	$\bar{d}$/kg dm^{-3}	H_1/kg mol^{-1}	H_2/kg mol^{-1}	V_1/dm^3 mol^{-1}	V_2/dm^3 mol^{-1}	V_0/dm^3 mol^{-1}
0.0006	0.0250	0.313 ± 0.003	0.0016 ± 0.00002	0.053 ± 0.024	0.1982 ± 0.002	1.007883 ± 0.00002	4.115 ± 0.002	0.345 ± 0.002	10.5 ± 0.3	1.69 ± 0.04	0.01807 ± 0.0008
0.0006	0.0499	0.189 ± 0.006	0.000 ± 0.001	0.21 ± 0.06	0.2260 ± 0.007	1.016280 ± 0.00001	4.269 ± 0.001	0.331 ± 0.001	10.4 ± 0.2	1.71 ± 0.06	0.01807 ± 0.0011
0.0006	0.0750	0.1196 ± 0.0015	0.0006 ± 0.0004	0.26 ± 0.02	0.2261 ± 0.006	1.024540 ± 0.00002	4.24 ± 0.09	0.308 ± 0.015	10.6 ± 0.2	1.77 ± 0.08	0.01849 ± 0.0017
0.0006	0.1251	0.0495 ± 0.0004	0.0010 ± 0.0001	0.52 ± 0.01	0.2390 ± 0.002	1.041792 ± 0.000009	4.026 ± 0.009	0.367 ± 0.001	10.6 ± 0.3	1.68 ± 0.08	0.01813 ± 0.0013

of macroscopic thermodynamic quantities.[24] We will refer to the first as excluded volume model and the second as the thermodynamic model. Both models suffer of approximation, and thus limitations.

The thermodynamic approach is approximate because it neglects the cross contribution to the flow of a generic component, assuming that both cross-term Onsager coefficients are nulls, bringing us to the following equation:

$$D_{ij} = \frac{c_i(\bar{V}_j - \bar{V}_0)}{1 - c_j(\bar{V}_j - \bar{V}_0)} D_{ii} \tag{5}$$

The model holds exactly only for ideal systems, and thus it is not rigorously applicable to nonideal mixtures. Actually, the derivation of the thermodynamic model is based on a approach used in 1970 by Vitagliano and Sartorio. Unfortunately, any rigorous application of a thermodynamic approach to mutual diffusion requires the activity coefficients of the solutes. These data are not very abundant in the literature for ternary systems, and they are usually affected by large errors.

The excluded volume model was introduced in order to predict ternary data from binary ones.[23] Since *n*-component systems require the knowledge of $(n-1)^2$ diffusion coefficients at any composition (eq 1), it would be desirable to evaluate the D_{ii} from the data of the corresponding binary systems, D_i, and then the D_{ij} from the D_{ii}. The "excluded volume" model was studied to be applied to systems containing macromolecules and without specific interactions between solutes, in cases where hydrodynamic volumes of the diffusing species are generally larger or much larger than the volume of solvent molecules themselves. In the excluded volume model, the solution is pictured as a dispersion of hard spheres in a continuum solvent, for which

$$D_{11} \approx D_1 \times \frac{\eta_1}{\eta_{12}} \tag{6}$$

$$D_{21} = D_{22} \times \frac{V_1 c_2}{(1 - V_1 c_1)^2} \tag{7}$$

where D_1 is the binary diffusion coefficient at c_1, η_1 is the binary viscosity at c_1, η_{12} is the ternary viscosity at (c_1, c_2), and V_1 is hydrodynamic volume. In such a simplified model, D_{ij} values are rigorously nulls only for uncharged, point particle components. Because of the not rigorous microscopic approach, in the excluded volume model the D_{ij} values so calculated do not respect the Onsager reciprocal relation (ORR). Analogously, this happens to other microscopic models applied to prediction of mutual diffusion coefficients.[25,26] eq 6 works the better the bigger is the size mismatch between components 1 and 2, i.e., when the correction of the "mixed solvent" viscosity is more appropriate. Equation 7 works well with solvent more abundant and faster than solutes, namely in cases where the counter-flow associated with the motion of the solute molecules is totally compensated for by the solvent, as experimentally proved in several macromolecular systems.[27,28] Consequently, the excluded volume model can be used in dilute solutions of solutes that present only nonspecific volume interaction and that have an effective molar volume larger or much larger than the solvent's molar volume. Therefore, the excluded volume model is a semiempirical and not a rigorous approach, but it is readily applicable to the class of systems of interest in biology.

Herein, we present an extended test on the predictive efficiency of the two approaches. Using a large number of

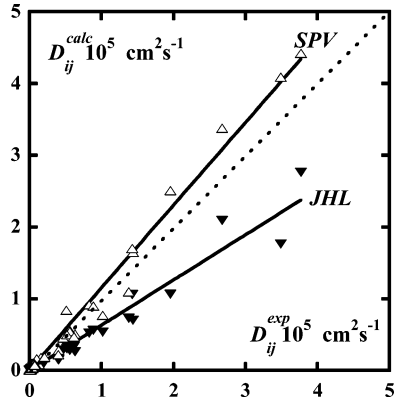

Figure 1. Correlation graph between 80 calculated and experimental mutual diffusion coefficients, corresponding to 10 multicomponent systems. The correlation between experimental and calculated mutual diffusion coefficients is reported for the two different prediction of D_{ij} (the thermodynamic, JHL (▼), and excluded volume, SPV (▽), models). The dotted line represents the perfect agreement between experiments and theory.

mutual diffusion data, about 80 (drawn in Figure 1), extracted from 10 ternary systems, we obtained the following correlation between experimental and calculated mutual diffusion coefficients: 1.13 ± 0.2 for the excluded volume model and 0.68 ± 0.01 for the thermodynamic one.

Finally, a cautious use of the excluded-volume or of the thermodynamic model is suggested, considering that both are approximated and that they must be applied to systems with different characteristics. In particular, the more similar the component sizes and natures are (as in ideal mixtures), the more the thermodynamic model appears to be applicable. In contrast, as proved by the extended test herein presented, the more different the component sizes or the higher the nonidealities are, the more preferable the excluded volume model should be.

IV. Results and Discussion

This paper reports both volumetric and mutual diffusion data on the ternary system lysozyme(1)−PEG2000(2)−water(0) at a constant protein concentration, $c_1 = 0.600$ mM and at several PEG2000 concentrations, $c_2 = 0.0250$, 0.0499, 0.0750, and 0.1251 M.

The partial molar volume of all the three components is almost constant within the experimental error in the range of PEG concentration explored (Table 1). The general small change of the $\bar{V}_i$ seems to confirm the absence of significant solute−solute specific interactions.

IV.a. Influence of PEG Molecular Weight on Protein Diffusion. The experimental diffusion coefficients of lysozyme(1)−PEG2000(2)−water(0) system, compared to those of lysozyme(1)−PEG400(2)−water(0)[14] system are shown in Figures 2−5 vs the PEG concentration, c_2. For the prediction of D_{ij} via eqs 6 and 7, we used a protein "effective" hydrodynamic volume, V_1, equal to 16.2 dm^3 mol^{-1}, as estimated in a previous paper.[29]

***D*$_{11}$.** The main diffusion coefficient for lysozyme, that links the lysozyme diffusion flow to its own concentration gradient, has a similar trend on PEG concentration for both systems (Figure 2). D_{11} values decrease as the PEG concentration increases, thus as the dielectric constant decreases and the medium viscosity increases. The difference between experimental and predicted D_{11} values, systematically overestimating the experimental ones, could indicate the presence of other secondary effects in determining the values of the lysozyme main diffusion coefficient. However it is interesting to point

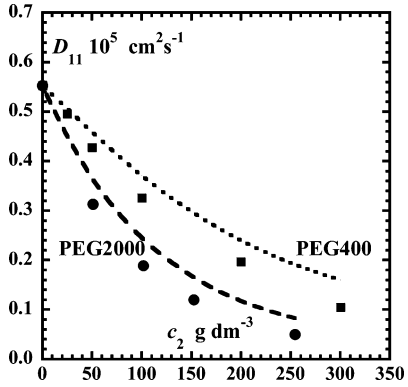

Figure 2. D_{11} experimental data (circles) and calculated values from eq 6 (lines) as a function of the PEG concentration. Comparison between the two ternary systems lysozyme(1)−PEG2000(2)−water (●) and lysozyme(1)−PEG400(3)−water (■).

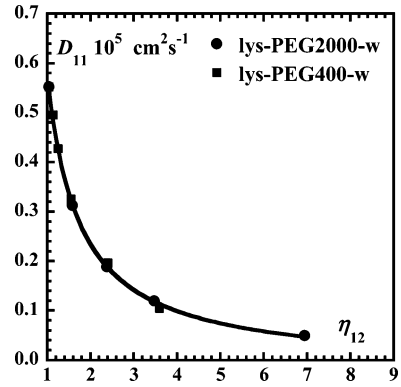

Figure 3. D_{11}, experimental data (circles) and calculated values from eq 6 (lines), as a function of the experimental relative viscosity. Comparison between the two ternary systems lysozyme(1)−PEG2000(2)−water (●) and lysozyme(1)−PEG400(3)−water (■).

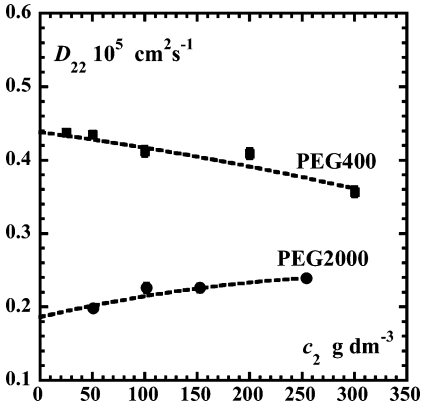

Figure 4. D_{22}, experimental data (circles) and calculated values from eq 6 (lines). Comparison between the two ternary systems lysozyme(1)−PEG2000(2)−water (●) and lysozyme(1)−PEG400(3)−water (■).

out that reporting the D_{11} as a function of the medium viscosity, a single curve fits the diffusion data for both systems, containing PEG400 and PEG2000 (Figure 3). Since the two PEG400 and PEG2000 aqueous solutions have different dependence of the dielectric constant on medium viscosity,[28] the uniform trend in Figure 3 suggests that the medium viscosity has the predominant effect in determining the D_{11} values.

***D*$_{22}$.** The PEG main diffusion coefficient links the PEG diffusion flow to its own concentration gradient. The PEG main diffusion coefficient D_{22} cannot be predicted using medium viscosity (Figure 4), because lysozyme−water cannot be considered as a continuum mixed solvent with respect to the PEG diffusing molecules, due to the large size of lysozyme

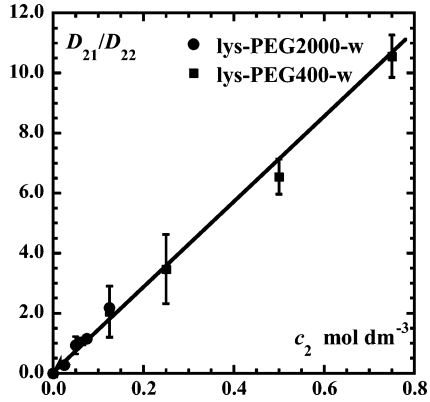

Figure 5. D_{12}/D_{11}, experimental data (circles) and calculated values from eq 7 (lines). Comparison between the two ternary systems lysozyme(1)−PEG2000(2)−water (●) and lysozyme(1)−PEG400(3)−water (■).

molecule. However it is to point out that the D_{22} values, for both systems, are practically coincident, within the experimental error, to the correspondent D_2 values of the binary system PEG−water. This indicates that presumably the obstruction effect of lysozyme (0.6 mM) on the motion of PEG molecules is irrelevant because of the very small protein volume fraction. The concentration dependence of the mutual diffusion coefficient of PEG with different molecular weights (e.g., PEG400 and PEG2000) in water is very different.[21] The PEG400 diffusion coefficients in water[21] and in lysozyme−water[14] systems decreases as the polymer concentration increases. Vice versa, the peculiar increasing trend of PEG2000 diffusion coefficients has already been discussed in detail elsewhere[21] in terms of a thermodynamic nonideality of PEG2000 solutions. The same discussion applies to D_{22} at increasing c_2 for lysozyme−PEG2000−water system.

***D*$_{21}$.** This coefficient relates the PEG flow to the protein concentration gradient. It is zero, by definition, at zero PEG concentration, then it increases very sharply at increasing c_2, reaching values larger than both main-term diffusion coefficients. D_{21} values evaluated from eq 7 lead to a good agreement between experimental and predicted values, both for PEG400 and 2000. In fact, the linear trend of the ratio D_{21}/D_{22}, drawn in Figure 5, proves the goodness of eq 7.

***D*$_{12}$.** This coefficient links the protein flow to PEG2000 concentration gradient. D_{12} values cannot be estimated by eq 7, but they are always very small and can be considered almost zero within the experimental error (see Table 1).

Altogether, these results, also supported by data relative to another systems containing PEG2000 and a different protein (human serum albumin, unpublished data), allow us to reach the following conclusions. In a ternary protein−PEG−water system, without specific protein−polymer interactions and at low protein concentration:

1. The protein main term diffusion coefficient can be reasonably well evaluated from the corresponding binary diffusion coefficient and from known system viscosity via eq 7.

2. The polymer main term diffusion coefficient is very close to the corresponding binary diffusion coefficient.

3. The diffusion coefficient relative to the motion of polymer under the protein concentration gradient can be efficiently evaluated from known polymer diffusion coefficient in the corresponding binary solution via eq 7.

4. The diffusion coefficient relative to the motion of protein under the polymer concentration gradient is almost zero.

These findings are useful in describing the diffusive process involved in many interface processes, such as protein crystallization (see below).

IV.b. Lysozyme Salting out Induced by PEG. It is possible to obtain, from a combination of these diffusion coefficients (nonequilibrium properties) and volumetric data, further information on the thermodynamics of the analyzed systems. This is a rare case in which from nonequilibrium properties, it is possible to extract equilibrium properties. In particular, it is possible to estimate the derivative of the chemical potential of a component with respect to the concentration of the other component. Details of the procedure are developed and described in previous works, where it was applied to lysozyme−salt[12,15] and to lysozyme−PEG400[14] aqueous solutions. Starting from the Onsager reciprocal relations (ORR) and from the Euler relations for the Gibbs free energy, we obtain two equations connecting the four derivatives of the chemical potentials, $\mu_{ij} = (\partial\mu_i/\partial c_j)$ with $i, j = 1, 2$, and the mutual diffusion coefficients in the solvent fixed reference frame, ${}_0D_{ij}$, and the partial molar volumes of the two solutes. These equations can be rearranged as

$$\mu_{21} = \frac{\mu_{22}[{}_0D_{11}(c_2\bar{V}_1) - {}_0D_{21}(1 - c_2\bar{V}_2)] - \mu_{11}[{}_0D_{11}(c_1\bar{V}_2) - {}_0D_{12}(1 - c_2\bar{V}_2)]}{{}_0D_{11}(1 - c_1\bar{V}_1) - {}_0D_{22}(1 - c_2\bar{V}_2)}$$

$$\mu_{12} = \frac{-\mu_{22}[{}_0D_{22}(c_2\bar{V}_1) - {}_0D_{21}(1 - c_1\bar{V}_1)] + \mu_{11}[{}_0D_{22}(c_1\bar{V}_2) - {}_0D_{12}(1 - c_1\bar{V}_1)]}{{}_0D_{11}(1 - c_1\bar{V}_1) - {}_0D_{22}(1 - c_2\bar{V}_2)} \quad (8)$$

where the cross derivatives are expressed as a function of the main ones. Therefore, if μ_{11} and μ_{22} values can be reasonable estimated, it is possible to get reasonable values for the cross derivatives μ_{ij}. In the case of the analyzed systems, we can assume that the derivative of the chemical potential of PEG with respect to its own concentration in the PEG2000−water and PEG400−water binary systems do not vary very much if we add lysozyme at a very low concentration as in our case ($c_1 = 0.0006$ mol dm^{-3}). This assumption, justified on the basis of the small protein concentration and of the absence of lysozyme−PEG specific interactions, allows to approximate the ternary μ_{22} value to that of the binary one, μ_2, calculated using literature activity data for PEG−water systems at 25 °C.[30] The estimation of the μ_{11} derivatives has been obtained as discussed elsewhere.[14] Anyway, the accuracy of this estimation is not so much important in determining the cross derivatives, μ_{12} and μ_{21}, because the factors that multiply μ_{11}, in eqs 8, are very small. Since $(c_1\bar{V}_2)$ and ${}_0D_{12}$ are almost zero eqs 8 can be rewritten

$$\mu_{21} \cong \frac{\mu_{22}[{}_0D_{11}(c_2\bar{V}_1) - {}_0D_{21}(1 - c_2\bar{V}_2)]}{{}_0D_{11}(1 - c_1\bar{V}_1) - {}_0D_{22}(1 - c_2\bar{V}_2)}$$

$$\mu_{12} \cong \frac{-\mu_{22}[{}_0D_{22}(c_2\bar{V}_1) - {}_0D_{21}(1 - c_1\bar{V}_1)]}{{}_0D_{11}(1 - c_1\bar{V}_1) - {}_0D_{22}(1 - c_2\bar{V}_2)} \quad (9)$$

μ_{21} values do not have such a biochemical interest, whereas μ_{12} values, expressing the effect of the PEG upon the lysozyme chemical potential, represents the PEG tendency to bring lysozyme toward precipitation and it is of interest in all the crowding phenomena. To evaluate the effect of the PEG molecular weight on the μ_{12} values, these have been computed deriving the protein chemical potential with respect to polymer concentration, expressed in g dm^{-3}. The obtained values are

Table 2. Thermodynamic and Gravitational Stability Parameters Derived for the Systems Lysozyme−PEG2000−Water (a) and Lysozyme−PEG400−Water (b) $\Delta c_2/\Delta c_1$ Represents the Percentage of the Concentration Plane in Which Gravitational Stability Exists

(a) PEG2000

c_1/mol dm^{-3}	c_2/mol dm^{-3}	$\Delta c_2/\Delta c_1$	(μ_{11}/RT)/dm^3 g^{-1}	(μ_{22}/RT)/dm^3 g^{-1}	(μ_{21}/RT)/dm^3 g^{-1}	(μ_{12}/RT)/dm^3 g^{-1}	(μ_{21}^{Lek}/RT)/dm^3 g^{-1}
0.0006	0.0250	95.47%	0.130	0.057			
0.0006	0.0500	98.02%	0.130	0.048	0.0128	0.0896	0.030
0.0006	0.0750	88.33%	0.130	0.046	0.0035	0.0179	0.030
0.0006	0.1251	88.74%	0.132	0.045	0.0030	0.0121	0.030

(b) PEG400

c_1/mol dm^{-3}	c_2/mol dm^{-3}	$\Delta c_2/\Delta c_1$	(μ_{11}/RT)/dm^3 g^{-1}	(μ_{22}/RT)/dm^3 g^{-1}	(μ_{21}/RT)/dm^3 g^{-1}	(μ_{12}/RT)/dm^3 g^{-1}	(μ_{21}^{Lek}/RT)/dm^3 g^{-1}
0.0006	0.0625	99.57%	0.130	0.044			
0.0006	0.1250	99.90%	0.130	0.025	0.0903	3.078	0.093
0.0006	0.2500	98.36%	0.130	0.016	0.0072	0.232	0.093
0.0006	0.4999	97.68%	0.132	0.011	0.0039	0.087	0.093
0.0006	0.7501	97.91%	0.132	0.010	0.0045	0.097	0.093

reported in Table 2 for two systems, lysozyme−PEG2000−water and lysozyme−PEG400−water. Unfortunately, for both systems there are some compositions at which μ_{ij} are affected by very large errors, therefore we have only three μ_{12} values reported in Table 2 for each system.

Since we are far form the solubility conditions we cannot integrate μ_{12}, to obtain μ_1 vs c_2, and thus obtaining further information on the PEG-induced lysozyme precipitation, as previously done for lysozyme−NaCl−water.[12] Moreover, the limited number of experimental data does not permit to obtain a reliable interpolating polynomial for μ_{12} as a function of c_2. For this reason we will limit the discussion to the μ_{12} values and their trend as a function of c_2. The μ_{12} for the two systems are reported as a function of the PEG concentration (expressed as g dm^{-3}) in Figure 6. All the calculated μ_{12} values are positive,

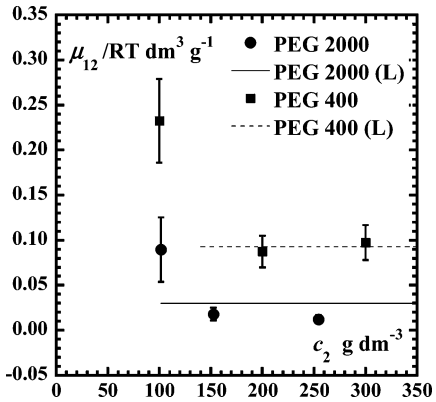

Figure 6. μ_{12}, experimental data (circles) and calculated values (L) from eq 10 (lines). Comparison between the two ternary systems lysozyme(1)−PEG2000(2)−water (●) and lysozyme(1)−PEG400(3)−water (■).

thus indicating that PEG addition increases the protein chemical potential. This is in perfect agreement with the Timasheff definition of PEG as a salting-out destabilizer.[31] The experimental μ_{12} data relative to lysozyme−PEG400−water system result systematically larger than the corresponding values of lysozyme−PEG2000−water system of a factor $M_{w,PEG2000}/M_{w,PEG400}$. This indicates that, in the case of lysozyme, the salting out effect at the same PEG concentration (expressed in g dm^{-3}) can be larger for PEG samples at low molecular weight. It is worth noting that an anomalous trend of the effect of PEG molecular weight were also observed for protein association rate, and it was eventually discussed in terms of a coillike behavior of larger PEG, opposed to the short PEG400, which is still in an extended structure.[32] Interestingly, at high PEG concentration, the experimental μ_{12} values coincide with those obtained by the Lekkerkerker's model.[33] In this model, the volume excluded by PEG to the protein, treated like a pair property, is considered a perturbation to the Helmoltz free energy of the binary protein solution. According to this model, the derivative of the protein chemical potential is simply correlated to the volume excluded by PEG molecules to the protein and can be expressed by the relation

$$\mu_{12} = RT \times \frac{\left(\frac{r_1 + r_2}{r_1}\right)^3 \times V_1}{1 - \left(\frac{r_1 + r_2}{r_1}\right)^3 \times V_1 c_1} \qquad (10)$$

where r_1 and r_2 are the radius of lysozyme and PEG molecules, respectively. Equation 10 shows that, in the Lekkerkerker derivation, the μ_{12} derivatives are independent of the PEG concentration, thus, in the graphic of Figure 6, they appear like straight horizontal lines.

IV.c. Protein Crystallization Induced by PEG. The above results are useful in analyzing the protein crystallization process. In fact, an analysis of the transport phenomena during the crystal growth requires the integration of the Fick's flow equation in a wide solute concentration range. Therefore, the knowledge of the diffusion coefficients as a function of the system composition is required. Large values of the cross-term diffusion coefficients, as those herein reported, can significantly affect the description of the mass transport in crystallization reactors,[16] and can modify the conclusions of theories on the effect of transport on the crystallization steps. Therefore, the multicomponent approach should be preferred to the usual pseudobinary approach. The predictive eqs 6 and 7 should stimulate such a novel approach.

In general, the increase of the polymer molecular weight slackens the protein diffusive response to its own concentration gradient, favoring protein sovrasaturation and then nucleation phenomena. Indeed, large fluctuations (by as much as 80%) in growth rate, in vicinal slope and in tangential velocity originating from coupling of bulk transport with nonlinear interface kinetics have been numerically predicted and experimentally observed.[34] The step bunch kinetics causing striations in crystals is governed by the kinetic Peclet number, a system-dependent parameter measuring the relative weight of bulk transport and interface kinetics. In a mixed kinetic-bulk system, crystal quality is expected to be less good under reduced gravity conditions.[34] Despite the fact that this theory has still to be extended to a multicomponent approach (including D_{ij}), its results are still useful. Indeed, this can be particularly relevant in the crystallization technique called free interface diffusion (FID).[35] During the FID crystallization process, diffusion giant fluctuation can

occur, that can induce gravitational instability at the interface between protein solution and precipitant solution. The knowledge of the diffusion tensor herein determined permits the analysis of gravitational stability. The occurrence of fingering and overstability phenomena depends on the range of Δc_1 and Δc_2 values across the diffusion boundary. An analysis of the gravitational stability conditions for the systems lysozyme−PEG−water can be done.[14] Results of this analysis are collected in Table 2, showing a slight reduction of the stable area in the Δc_1 and Δc_2 plane at increasing concentration of PEG. Analogous results are obtained also for the lysozyme−PEG400−water, herein correcting a previous result.[14]

Protein concentration gradients produce a PEG cross-flow that increases with the polymer molecular weight. Then, protein main flow is always accompanied by PEG cross-flow in the same direction, in a process that could favor conditions of protein sovrasaturation. In fact, in the event of protein crystal formation, the presence of a longer PEG sample produces a higher number of PEG molecules dragged by a protein molecule (D_{21}/D_{11}) and a lower values of D_{22}, allowing a greater polymer supply in the surroundings of the crystal.

V. Conclusions

The analysis of multicomponent diffusion on lysozyme-poly(ethylene glycol)−water solutions provides insights on (i) the predictive ability of protein mutual diffusion in crowded solutions, (ii) the salting out effect of PEG at different molecular weight, and (iii) the hydrodynamics in solutions where protein crystals grow.

A theoretical model based on exclusion concepts works well in predicting protein and polymer diffusion, therefore the multicomponent approach in describing mass transport of macromolecules can be suitably used. The combination of D_{ij} and Onsager reciprocal relation allows the determination of PEG induced salting out of lysozyme. These data exhibit an interesting effect of PEG molecular weight (PEG400 induces a salting out higher than PEG2000, when referred per gram of PEG). Relevant hydrodynamic effects of PEG on protein crystallization appear to be due to both main and cross-flows. The increase of solution viscosity reduces all flows, stabilizing the supersaturation condition during nucleation and a more regular adhesion of the protein molecules in the growth. The number of polymer molecules "dragged" by a protein molecule may reach very high values, ensuring a continuous supply of precipitant in the surroundings of growing protein crystals.

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MA0605705