

Net Charge and Electrophoretic Mobility of Lysozyme Charge Ladders in Solutions of Nonionic Surfactant

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We report on the electrophoretic mobility and on the thermal diffusion of lysozyme proteins dissolved in aqueous solutions of a nonionic surfactant ($C_{12}E_6$) at a wide range of concentrations of the surfactant (0–20% by weight). We want to estimate the influence of a dense network of elongated micelles of $C_{12}E_6$ on the effective charge of the proteins as observed in the capillary electrophoresis experiments. The possible mechanism leading to the change in the effective charge of protein could involve the deformation of the cloud of counterions around the protein when it squeezes through the narrow (of the order of a protein diameter) aqueous channels formed in the solution of elongated micelles. The combination of independent measurements of the electrophoretic mobility of a family of modified proteins (lysozyme charge ladder [Colton et al. *J. Am. Chem. Soc.* **1997**, *119*, 12701]), of the microviscosity of the solutions of surfactant (obtained via fluorescence correlation spectroscopy), and of the hydrodynamic radius of the proteins (photon correlation spectroscopy) allow us to conclude that the effective charge of the proteins is not affected by the presence of surfactant, even at high concentrations.

Introduction

In living cells, various macromolecules and lipids often form a crowded environment of polymeric or supramolecular networks of concentrations reaching several hundred grams per liter. These networks can occupy up to 40% of the available volume.^{2,3} Yet, in typical in vitro assays, the activity of proteins is studied in neat water or in a low-viscosity aqueous buffer. This practice overlooks a number of queries, answers to which—in principle—are not known. These include the uncertainty of how (and if) the net charge of the protein, its activity, rate of association, and enzymatic catalysis are affected by the supramolecular environment. An additional source of ambiguity is the lack of detailed knowledge of the polymeric structure of living cells. Since in many biological processes proteins diffuse between lipid membranes, i.e., in endoplasmic reticulum,⁴ mitochondria,⁵ or near the nucleus envelope, we believe that there is a need to study the details of the behavior of proteins in crowded environments. In this paper, we explore the microrheology of proteins and their net charge in a model—and well-known—micellar fluid. The choice of a well-known system of an aqueous solution of the nonionic $C_{12}E_6$ surfactant provides for a detailed knowledge of both the chemical composition of the network and of the distribution of obstacles in space.

Crowded environments can strongly influence the stability of proteins and their affinities for ligands. Davis-Searles⁶ and O'Connor⁷ demonstrated that nonionic additives (fructose and sucrose) increased the stability of native conformations of proteins. Nonionic surfactants can induce a partial breakdown

of the globular conformation of proteins, e.g., legumin in PGE (a mixture of stearic and palmitic acid esters).⁸ Interactions of PGE and legumin lead to the disruption of internal bonds in the protein and—consequently—to partial unfolding. Such strong interactions with nonionic additives are not a rule for all proteins. For example, albumin in a nonionic poly(oxyethylene)isooctyl phenyl ether showed little interaction with the surfactant,⁹ while it interacted strongly with anionic and cationic surfactants sodium dodecyl sulfate and centrimethylammonium bromide. Interactions of proteins with charged surfactants are complex and show a strong interplay between hydrophobic and electrostatic interactions.^{8–12} In this paper, we study the net charge of the lysozyme protein and its conformation in an environment of micelles formed by a nonionic surfactant. We use three techniques—capillary electrophoresis, fluorescence correlation spectroscopy, and photon correlation spectroscopy—to determine the net charge of proteins in surfactant solutions, the microviscosity of the proteins experienced in their thermal motion, and the hydrodynamic radius of the proteins.

The net charge of proteins is determined both by their sequence of aminoacids and by the structure and properties (e.g., pH) of the solvent.¹³ There are various methods for the determination of the net charge of a protein. For example, the technique of proton titration measures the number of protons bound to the protein;¹⁴ sequence analysis provides data on the number of groups that can dissociate (ionize) at a given pH of the solution; and¹⁴ finally, capillary electrophoresis and especially the technique of the protein charge ladder allows for measurement of electrophoretic mobilities and—provided one correctly knows the viscosity of the solution—the charge of the proteins.^{13,15–17} The basic idea of capillary electrophoresis is that in this technique the applied electric field acts on the net charge of the protein and in the first approximation the mobility

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is not affected by screening: the counterions that surround the charged protein do not influence the force, $F = Eq$, exerted by the electric field E on a protein of net charge q . This force drags the proteins at velocity, $v = qE/(6\pi\eta R)$, where η is the viscosity of the solution and R is the hydrodynamic radius of the protein. This simplified description contains the salient feature of the capillary electrophoresis in that the method yields the value of q provided that the viscosity and the hydrodynamic radius are known. However, as discussed later, the full description of electrophoretic mobilities requires solving the nonlinear Poisson–Boltzmann equation (distribution of ions and screening effects) and equations of hydrodynamics to account for the flow of the ions under the external electric field.¹⁷

Here, we use the lysozyme charge ladders in basic solutions (pH = 8.4). At this value of pH, a lysozyme is positively charged. Lysozyme charge ladders are formed by the controlled neutralization (acetylation) of charged (e.g., amine) groups at the surface of the protein.¹³ There are six amino groups that, after acetylation, present no charge. An incomplete acetylation results in a statistical mixture of proteins with a varying number $n = 0, 1, 2, \dots, 6$ of acetylated groups and—equivalently—with a varying charge $q^{(n)} = (Z_0 - n\Delta Z)e$. On the basis of the amino acid sequence of the lysozyme, one expects $Z_0 \approx 8$ and $\Delta Z \approx 1$, where e is the charge of a proton. In general, because of charge dissociation at chemical equilibrium, Z_0 and ΔZ need not be integers.¹³ The number of proteins of a given charge (the distribution of n 's) in the mixture depends on the details of the reaction protocol. Optimizing the reaction conditions, we obtained a mixture with an almost uniform distribution of the number of acetylated groups. In principle, different groups at the surface of a lysozyme can possess a different effective charge; here, we adopt the approximation that each group contributes the same charge—the average charge per group. The advantage of electrophoresis on charge ladders over electrophoresis on a single variation of a protein is that it allows determination of Z_0 , the electrophoretic mobility of an unmodified protein, and the effective charge $q^{(0)}$, in one experiment, provided we know the viscosity experienced by the proteins and their hydrodynamic radii. Our experiments demonstrate that the technique of electrophoresis on charge ladders is suitable for the study of proteins in crowded environments. We supplement electrophoresis with a measurement of the hydrodynamic radius of the proteins via photon correlation spectroscopy (PCS)¹⁸ and a measurement of the coefficient of viscosity experienced by freely diffusing lysozyme proteins via fluorescence correlation spectroscopy (FCS).^{19–22} We use the combination of capillary electrophoresis (CE), PCS, and FCS data to analyze the electrophoretic mobility of proteins.

Materials and Methods

Surfactant Solution. We used aqueous solutions of the nonionic surfactant $C_{12}E_6$, dissolved in a phosphate (0.02 M, pH = 7, $I = 0.154$ M) and Tris–Gly (pH = 8.4, 25 mM Tris base, and 192 mM glycine) buffers. We set the ionic strength of the phosphate buffer to physiological value of 0.154 M by adding NaCl. We prepared solutions by weighting the appropriate quantities of components and by mixing them to homogeneity in a vial placed on a magnetic stirrer with a heat plate. We purchased the surfactant $C_{12}E_6$ (hexaethylene glycol monododecyl ether, catalog no. 52044, purity better than 98%) from Fluka and used it as delivered. Fluka reports the melting temperature T_M of pure $C_{12}E_6$ to be between 27 and 28 °C. We measured T_M via differential scanning calorimetry and obtained the value of 27.11 °C. The molecular weight of $C_{12}E_6$ is 450.66 g/mol.

The aqueous phase behavior of $C_{12}E_6$ has been extensively studied^{23,24} and is well-known.²⁵ At low concentrations and low temperatures (below 20 °C and below 0.1% of surfactant by weight), we expect our surfactant to self-organize into spherical micelles. At higher concentrations, micelles are expected to grow in length and eventually to form a network^{26–28} of intertwined highly elongated micelles at concentrations close to the isotropic hexagonal phase transition which occurs at 38% by weight of surfactant. Although the above expectations are for water–surfactant systems and we used a buffer instead of pure water, we have checked that in the solutions of $C_{12}E_6$ with phosphate buffer the transition temperatures and transition concentrations do not change appreciably in comparison to the $C_{12}E_6$ –water solutions.

Lysozyme and Lysozyme Charge Ladders. We purchased highly purified (by crystallization repeated three times and by dialysis) lysozyme protein from Sigma–Aldrich (catalog no. L7651). The tertiary structure of lysozyme is a prolate ellipsoid with the short and long axes of 30 and 50 Å, respectively (based on the crystallographic structure). For fluorescence correlation spectroscopy, the lysozyme was labeled with the TAMRA (i.e., 5(6)-carboxytetramethylrhodamine *N*-hydroxysuccinimide ester) fluorescent dye with the absorption and emission in the region of 555 and 580 nm, respectively. We purchased TAMRA from Sigma–Aldrich (catalog no. C4759). For capillary electrophoresis and photon correlation spectroscopy, the charge ladder has been prepared. The preparation of the charge ladder^{13,15–17} was done according to the following recipe: The protein solution used was the 5 mL of the 2 mM lysozyme solution in the carbonate buffer (50 mM, $I = 100$ mM) of pH 10. The solution of acetic anhydride (200 mM) in dioxane was used as an acetylation agent. The reaction was started by addition of 0.3 mL of the solution of acetic anhydride (200 mM) in dioxane to the 5 mL of lysozyme solution (2 mM and pH 10). The reaction was terminated after 25 min by the addition of 13 mL of the Tris–glycine buffer (25 mM Tris, 192 mM Gly). Because we did not allow the reaction to proceed to completion, we obtained molecules of lysozyme with one, two, three, four, five, and six acetylated groups, i.e., groups with one, two, three, four, five, and six neutralized groups that otherwise (if not acetylated) could present a positive charge. From now on, we refer to the set of derivatives of lysozyme with the same number of neutralized groups—thus, the set of molecules that possess the same net charge—as a “rung” in the ladder. A successful preparation of the charge ladder is one which makes the concentration of each of the rungs roughly equal. We checked the intensity of each of the rungs directly by capillary electrophoresis (CE); the height of a peak corresponding to a given rung in CE is proportional to the abundance of this rung (its concentration) in the mixture. The above recipe yielded a solution with similar concentrations of all the rungs.

Photon Correlation Spectroscopy. We carried out the light scattering experiments using a Zeiss He–Ne laser HNA 188-S source, operated at $\lambda = 632.8$ nm, 50 mW, and linearly polarized light. The scattered light was collected using a monomode optical fiber mounted on the arm of a goniometer (ALV/SP-125, ALV GmbH, Langen, Germany) and fed into a high quantum efficiency photon counting avalanche photodiode (APD unit). The angle θ between the incident beam and the axis of the goniometer was changed between 30° and 150°. Thus, the scattering wave vector q given by the relation $q = [4\pi n_R \sin(\theta/2)]/\lambda$, with λ being the wavelength and n_R the sample refractive index (water), varied in the range from 6.8×10^6 to 2.51×10^7 1/m. The capillary was mounted on a special

capillary holder at the center of a temperature-regulated tank. The temperature in the tank was controlled with a 0.1 °C resolution via a recirculation from a large, temperature-stabilized water bath. In order to establish the time autocorrelation function of the scattered signal, we processed the measured photocurrent with a PC-controlled, 256-channel, 4700-Malvern correlator. This machine was able to work in one of two modes—either a linear or a logarithmic one—with sampling times as short as 0.05 μ s. The relaxation times were obtained from the exponential decay of the lysozyme concentration autocorrelation function, i.e., $\exp(-t/\tau_{\text{PCS}})$; we were able to measure relaxation times, τ , in the range from 10^{-5} to 10^3 s. We used the dependence of the relaxation time on the wave vector $\tau_{\text{PCS}} = 1/Dq^2$, to establish the coefficient of diffusion of the proteins.

Fluorescence Correlation Spectroscopy. In the FCS experiments, information about the coefficient of diffusion of various molecules can be inferred from the recordings of the intensity of fluorescent light emitted from a small, optically defined focal volume. The intensity of the I signal fluctuates as the fluorescently labeled protein diffuses in and out of the focal volume or as the molecule changes its photophysical properties. The focal volume is of the order of 1 fl (i.e., 10^{-15} L), and the concentration of the fluorescent molecules is in the nanomolar range, which combined make FCS a single-molecule technique of detection. Given the average time τ the molecule spends in the focal volume and the dimensions F of this volume, we can derive the self-diffusion coefficient, D , of the molecule ($F^2 \sim D\tau$). The evolution of the fluorescence intensity can be analyzed via an autocorrelation function which contains the information about the average number of fluorescent molecules in the focal volume, about their average residence time in the focal volume, and about their photophysical properties.^{18,19} We carried out single-color FCS measurements (autocorrelation) at 25 °C on a commercial system (ConfoCor II, Carl Zeiss, Jena, Germany) using—for excitation—a 543 nm He–Ne laser. We used a C-Apochromat 40 \times /1.2 (N.A. 1.2) objective with the pinhole diameter set to 78 μ m (1 Airy unit for 543 nm laser) for all measurements that we report here. For a detector, we used an avalanche photodiode. The laser intensities used did not exceed about 40 μ W (power density at the focal point ~ 30 kW/cm²). The accessible time range for the measurements of the autocorrelation function was 1 μ s–10 s. Distribution of the intensity (I) of the laser light in the focal volume is often approximated as a three-dimensional Gaussian:

$$I(x,y,z) = I_0 \exp(-2(x^2 + y^2)/F^2 - 2z^2/P^2)$$

where F is the cross-sectional length in the x – y plane and P is the height of the illuminated element of volume. We recorded the intensity $S(t)$ of fluorescence emitted from this volume as a function of time. This intensity fluctuated as single fluorescently labeled molecules diffused in and out of the focal volume (i.e., 10^{-15} L). The signal $S(t)$ allows the extraction of the distribution of residence times (τ) of the tracers in the focal volume by analyzing the autocorrelation function of $S(t)$: $g(\Delta t) = \int S(t)S(t + \Delta t) dt$. We calculated the coefficient for diffusion as $D = F^2/(4\tau)$ and the coefficient of viscosity, η , using the Einstein–Stokes formula $\eta = kT/(6\pi DR)$, where k is the Boltzmann constant, T is temperature in kelvin, and R is the radius of the probe. To exclude the possibility of the non-Gaussian shape of the confocal volume, free diffusion of rhodamine 6G in water was measured prior to each experiment, and the obtained data fit well with the single-component autocorrelation function for normal diffusion. The value of the fitted structure parameter $\omega = P/F$ for rhodamine 6G amounted to about 5. The diffusion

coefficient for rhodamine 6G at 20 °C is 2.8×10^{-6} cm²/s²⁹ that corresponds to a diffusive time τ of 31 μ s in our FCS setup. It gives the following dimensions of the focal volume: $F = 0.186(3)$ μ m, $P = 0.932(15)$ μ m. At least 20 correlation functions accumulated for 30 s were recorded for each solution and their average was analyzed with Origin in the nonlinear least-square fitting module. The standard deviation of the values of the autocorrelation function was calculated as the standard deviation of the average and used for the calculation of the χ^2 test.

Electrophoresis. We performed the experiments on a Beckman-Coulter P/ACE MDQ (Fullerton, CA) capillary electrophoresis system equipped with a PDA detector ($\lambda = 214$ nm).^{30,31} The solutions were made in Tris buffer with a pH of 8.4 (25 mM Tris base and 192 mM glycine). At this pH, lysozyme is positively charged ($pI \sim 11$), and thus, silicate capillaries—which are negatively charged at pH values larger than 2–3—were impractical as the oppositely charged proteins could adsorb onto the walls of the capillaries. In order to neutralize the negative charge on the walls of the capillaries, we coated them with a polycationic polymer—Polybren (hexadimethrine bromide). Such a noncovalent coating limits the adsorption of lysozyme onto the walls of the capillary. We purchased Polybren from Sigma Aldrich (catalog no. 107689). The process of coating the capillary is very simple: it is sufficient to rinse the capillary with a 7.5% (w/w) aqueous solution of Polybren for 15–20 min. In the case of a positively charged capillary wall, the separation with CE has to be carried on with the reversed polarity (– +) (cathode at the inlet and anode at the outlet of the capillary). We checked—by performing several repeated runs in the same capillary—that the Polybren coating of the walls of the capillaries was stable in buffer; Polybren was not removed by the flow of the buffer (the capillary was rinsed with the buffer between runs). It turned out, however, that the separation cannot be achieved when the capillary was filled with the C₁₂E₆ solution; the surfactant removed the Polybren coating. In order to circumvent this problem, we ran the capillary electrophoresis experiments on charge ladders in surfactant solutions in neutrally coated capillaries (eCAP neutral capillary, commercially available from Beckman). The total length, L_t , of the capillary was 59 cm, and the length of the capillary from the inlet to the detector, L_d , was 48.7 cm. The inner diameter of the capillary was 50 μ m. The capillary was thermostated in 25 °C. In the case of the neutrally coated capillary, the electroosmotic flow is zero which allowed us to calculate the electrophoretic mobilities according to the following formula:

$$\mu = \frac{v_{\text{lys}}}{E} = \frac{\frac{L_d}{t_p}}{\frac{V}{L_t}} = \frac{L_t L_d}{V} \frac{1}{t_p} \quad (1)$$

where $V = 30$ kV is the applied voltage during the separation, v_{lys} is the lysozyme velocity, E is the electric field, and t_p is the migration time of lysozyme (time of flow).

Before each measurement (separation of the ladders in solutions of surfactant), we filled the capillary with the solution of surfactant. The time required for the surfactant solution to fill the capillary was calculated according to the Poiseuille equation (eq 2), taking into account the exponential growth of viscosity with surfactant concentration. The poiseuille equation gives the time, t , required for the certain volume, V_B , of liquid to flow through the cylindrical vessel (of radius r and length l)

under the constant pressure difference, Δp , applied to the ends of the vessel. The Poiseuille equation underlies the principle of determining the viscosity of liquids, by measuring the time of flow through the pipe of known dimensions (e. g., Cannon-type viscometer).

$$V_B = \frac{\pi r^4 \Delta p}{8\eta l} t \quad (2)$$

The time, t , required for the solution of surfactant to fill the whole capillary follows from the geometrical considerations. The volume of the capillary is about $1.16 \mu\text{L}$ ($L_c = 58 \text{ cm}$, $r = 25 \mu\text{m}$). The time of flow, t , is proportional to the viscosity of the solution. The highest concentration of surfactant used for the separation was 20% by weight, and the time required for filling of the capillary was more than 12 min. In the case of the solution of 30% of surfactant, the capillary should be rinsed with the surfactant solution for more than 1 h. Once the capillary was filled with the solution, we introduced the sample of the lysozyme charge ladder into the capillary by applying 0.034 bar of pressure (0.5 psi) for 3 s. The volume of the injected sample is of the order of 10^{-9} L ($0.001 \mu\text{L}$).

Theory

In capillary electrophoresis, the velocity of the flow of lysozyme of charge q is given by

$$v = qE/f \quad (3)$$

where f is the hydrodynamic drag. Assuming that the lysozyme is a sphere of radius R moving in a liquid of viscosity, η , the drag is given by the Stokes formula:

$$f = 6\pi\eta R \quad (4)$$

The corresponding quantity that depends only on molecular parameters is called electrophoretic mobility, μ , and is defined as follows:

$$\mu = \frac{v}{E} \quad (5)$$

Taking into account eqs 3 and 4, we obtain the simple formula for electrophoretic mobility in a simple fluid:

$$\mu = \frac{q}{6\pi\eta R} \quad (6)$$

In experimental studies, the macromolecular ions, such as proteins, are usually dissolved in buffer solutions containing small ions of opposite charge. In this case, eq 6 has to be modified to include the effects introduced by counterions surrounding the macromolecular ion. The external electric field acts on the macromolecular ion and surrounding counterions in opposite directions. This effect results in an additional force exerted on the macroion by the flow of the buffer ions in the immediate proximity of the protein. This additional force reduces the velocity of the macroion as compared to the one calculated with eq 6. Booth^{32,33} gave the equation for electrophoretic mobility of the spherical nonconducting, charged particle in the solution with nonzero charge density:

$$\mu = \frac{Ze}{6\pi\eta R (1 + \kappa R)} + \sum_{j=2}^{\infty} \frac{Z_j e^{2j-1}}{6\pi\eta R^j (\epsilon k T)^{j-1}} (X_j + Y_j + W_j) \quad (7)$$

where e is the unit charge (charge of the proton; $e = 1.602 \times 10^{-19} \text{ C}$); Z is the effective number of unit charges (such that $q = Ze$); and κ is the Debye–Hückel constant (8). The κ^{-1} is the Debye–Hückel screening length

$$\kappa = \sqrt{\frac{2e^2 N_A I}{\epsilon_0 \epsilon k T}} \quad (8)$$

Here, ϵ is the dielectric constant of the fluid, I is the ionic strength, k is the Boltzmann constant ($k = 1.38 \times 10^{-23} \text{ J/K}$), T is temperature in kelvin, N_A is the Avogadro number, and $\epsilon_0 = 8.85 \times 10^{-12} \text{ C}^2/(\text{N m}^2)$ is the permittivity of a vacuum. The function $f(\kappa R)$ from the above formula was given by Henry³⁴

$$f(\kappa R) = 1 + \frac{(\kappa R)^2}{16} - \frac{5(\kappa R)^3}{48} - \frac{(\kappa R)^4}{96} + \frac{(\kappa R)^5}{96} + \left(\frac{(\kappa R)^4}{8} - \frac{(\kappa R)^6}{96} \right) \exp(\kappa R) \int_0^{\kappa R} \frac{\exp(-x)}{x} dx \quad (9)$$

The values of the Henry function vary between 1 and 1.5, acquiring the highest value for the solutions with high values of κR ($\kappa R \sim 10^2$ or 10^3). The remaining terms (X_j , Y_j , W_j) from eq 7 are the functions of concentration, charge, and mobilities of the mobile ions in the solution. The terms X_j , Y_j , and W_j take into account the effect of the distortion of the equilibrium distribution of ions by the external electric field. Neglecting the nonlinear effects¹⁷ in the charge Ze simplifies eq 7 in the following way:

$$\mu = \frac{Ze}{6\pi\eta R} \frac{f(\kappa R)}{(1 + \kappa R)} \quad (10)$$

The above equation that relates the electrophoretic mobility to the molecular parameters is a relatively good approximation when nonlinear effects are absent.¹⁷ This equation does not take into account the distortion of the equilibrium distribution of the buffer ions around the macroion (protein) by the external electric field (see the standard model of electrokinetic properties of colloids).³⁵ In our work, eq 10 formed the basis for the determination of the net charge on the proteins from the electrophoretic mobility.

Protein Charge Ladders in a Buffer

A protein charge ladder is a set of derivatives of a protein that differ in charge but not in hydrodynamic radius R or conformation. Capillary electrophoresis often resolves this set of derivatives into a set of distinct peaks—the rungs of the ladder (Figure 1). On the basis of the observed times of emergence at the detector, we can calculate from eq 1 the electrophoretic mobilities of each rung of the charge ladder. The electrophoretic mobilities of the subsequent rungs of the charge ladder can be described by the following formula (see eq 10):

$$\mu^{(n)} = \frac{e(Z_0 - n\Delta Z)}{f} \frac{f(\kappa R)}{(1 + \kappa R)} = \mu_0 - \frac{e\Delta Z}{f} \frac{f(\kappa R)}{(1 + \kappa R)} n \quad (11)$$

Where, $\mu^{(n)}$ is the electrophoretic mobility of the n th rung of the charge ladder, $Z_0 e$ is the charge of the native protein, $\Delta Z e$ is the effective charge removed from the protein upon acetylation, n is the number of acetylated groups (amine groups), and f is the friction coefficient. For the case of the spherical

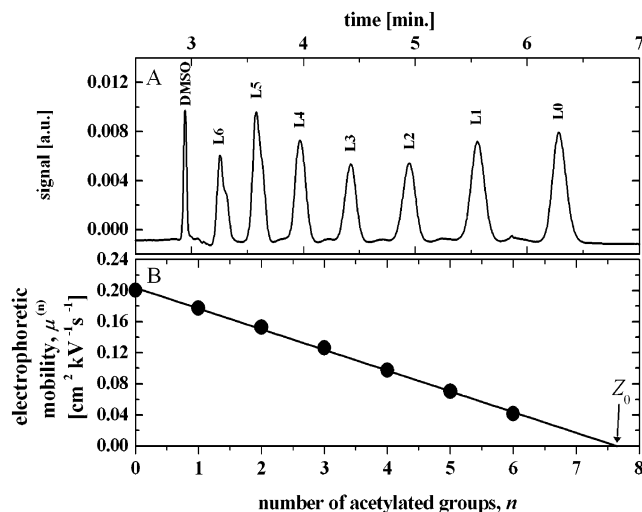


Figure 1. (A) Raw data (electropherogram) registered for the lysozyme charge ladders on the Polybren-coated capillary. The first peak corresponds to the neutral marker (DMSO) and gives the value of electro-osmotic flow (EOF). The following peaks correspond to the subsequent rungs of the lysozyme charge ladder, where L_n is the lysozyme with n amine groups blocked by acetylation. (B) Electrophoretic mobilities of the lysozyme charge ladder as a function of the number of acetylated amine groups on lysines. Each acetylation block is one amine group and decreases the charge of the lysozyme (the slope of the line is negative). The y-intercept gives the value of the electrophoretic mobility, $\mu^{(0)}$, of a native lysozyme; the x-intercept gives the charge, Z_0 , of a native lysozyme (when the charge Z_0 is removed from the protein, its electrophoretic mobility becomes 0). The slope of the line is a measure of the viscosity, η , of the solution.

particle, the friction coefficient is given by the Stokes formula ($f = 6\pi\eta R$), and we can write

$$\mu^{(n)} = \mu_0 - \frac{e\Delta Z}{6\pi\eta R} \frac{f(\kappa R)}{(1 + \kappa R)} n \quad (12)$$

Thus, the dependence of electrophoretic mobility on the number of acetylated groups is linear, with the slope, a , given by

$$a = - \frac{e\Delta Z}{6\pi\eta R} \frac{f(\kappa R)}{(1 + \kappa R)} \quad (13)$$

determining the ratio of $\Delta Z/\eta$ and the free parameter giving the value of the electrophoretic mobility, $\mu^{(0)}$, of the unmodified (native) protein. Extrapolating the observed dependence of residence times on the charge of the protein to the zero electrophoretic mobility (neutral protein), we can find the effective, net charge, Z_0 , of the native protein, expressed in terms of the number of the groups that need to be blocked to completely discharge the protein. Please note that if we place the same ladder in the surfactant solution we can get the relative viscosities from the ratio of the slopes,

$$\eta/\eta_0 = a(\phi = 0)/a(\phi) \quad (14)$$

where ϕ is the surfactant fraction by weight, under the assumption that the change in the concentration of surfactant does not change the hydrodynamic radius R or the charge change, ΔZe , introduced by acetylation.

An application of the above analysis (eqs 12 and 13) to the data shown in Figure 1 gives the following results: the mobility of native lysozyme $\mu^{(0)} = 0.204(2)$ [$\text{cm}^2/(\text{kV s})$], charge $Z_0 = 7.9 \pm 0.2$, and the slope of the line, $a = -0.0267(4)$ [$\text{cm}^2/(\text{kV s})$ per acetylation] (Figure 1). We show raw CE data in Figure 1A, and the mobilities, in Figure 1B. The value of the

$f(\kappa R)/(1 + \kappa R)$ correction for our system (Tris-Gly buffer with $\kappa = 0.21$ 1/nm and lysozyme, with $R = 1.9$ nm (see the next section), gives $\kappa R = 0.4$) amounts to about 0.72. We use this value to calculate the ratio of $\Delta Z/\eta$ according to

$$\frac{\Delta Z}{\eta} = - \frac{a6\pi R}{e} \frac{(1 + \kappa R)}{f(\kappa R)} \quad (15)$$

where the slope a is taken from Figure 1B. From (15), we get the value of $\Delta Z/\eta = 0.823$ [$1/(\text{mPa s})$]. In order to determine the value of ΔZ , we should know the viscosity of the buffer. The dynamic viscosity, η_0 , of the Tris-Gly buffer has been determined by measuring the kinematic viscosity, ν_0 , and density ρ_0 . Kinematic viscosity of the buffer was measured using a glass Cannon-type viscometer and the density of the buffer solution was measured using an Anton Parr densimeter. The obtained value of dynamic viscosity, $\eta_0 = \nu_0\rho_0$, was $\eta_0 = 0.930(2)$ mPa s.

We used eq 15 to calculate the change in charge, ΔZ , associated with a single acetylation, and—for ionic strength $I = 3.9$ mM—obtained a value of $\Delta Z = 0.77(4)$ in the units of elementary charge, e . This value is consistent with ΔZ reported in earlier work¹³ (ΔZ ranging from 0.86 for $I = 8$ mM to 0.98 for $I = 100$ mM). The net charge of lysozyme, $Z_0 \times \Delta Z$, can be now estimated to have the value of $+6.1 \pm 0.3$ (expressed in the units of the elementary charge, e). This value of net charge is lower than the value of the net proton charge, $Z_p \sim 6.8$ reported in the literature from the titration data.³⁶ In the range of pH around 8, the titration measurements differed in Z_p by 0.8.^{36,37} Taking this uncertainty into account, we conclude that our measurement of the net charge on the protein is consistent with earlier reports.

Lysozyme and Lysozyme Charged Ladders Diffusion in a Buffer

Determination of the net charge requires an independent measurement of the hydrodynamic radius R of lysozyme. A very reliable method for the determination of size is the photon correlation spectroscopy. In this technique, we measure the intensity–intensity correlation function of the scattered light.¹⁸ From the theory of PCS, it follows that the decay of this function for the solution of $m = 1, 2, 3, \dots, M$ different species each characterized by the diffusion coefficient D_m is exponential in time, t , i.e.

$$g^{(2)}(t) - 1 = \sum_m^M A_m \exp(-q^2 D_m t) \quad (16)$$

In principle, each of the derivatives in the charge ladder could have a different diffusion coefficient. We have measured the autocorrelation function for the lysozyme charge ladder in the Tris-Gly buffer. All data were fitted using a single-exponential function (see eq 16) for all rungs in a lysozyme charge ladder indicating that the lysozyme diffusion coefficient was independent from its charge in the Tris buffer (Figure 3). In this way, we also confirmed that the acetylation reaction does not change appreciably the conformation and consequently the hydrodynamic radius of lysozyme. As a reference for the diffusion coefficient determined for the lysozyme charge ladder, we used the diffusion coefficient of native lysozyme. We prepared the lysozyme solution in a buffer and determined the diffusion coefficient as a function of protein concentration. Extrapolating this concentration to zero gives us the single-particle diffusion coefficient of a lysozyme in a solution. In Figure 2, we show

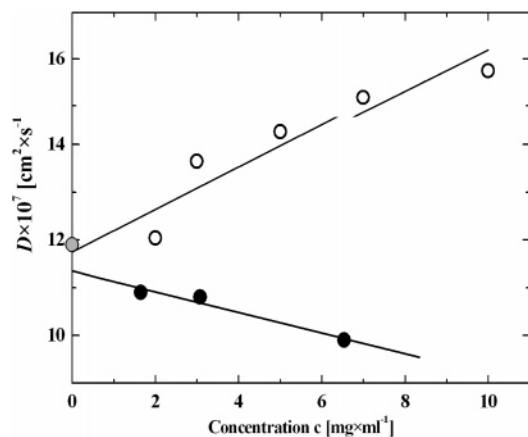


Figure 2. Diffusion coefficient of unmodified lysozyme as a function of lysozyme concentration in two different buffers: high ionic strength phosphate buffer ($I = 0.154$ mM; full black circles) and low ionic strength Tris-Gly buffer ($I = 4$ mM; empty circles). In the high ionic strength buffer, the electrostatic interactions are screened and we observe attraction between proteins. In the low ionic strength buffer, the proteins repel each other which results in the diffusion coefficient increasing with the concentration of the protein. The hydrodynamic radius is derived from the diffusion coefficient, D_0 , extrapolated to zero concentration of the protein. Literature data concerning the diffusion coefficient of lysozyme extrapolated to zero concentration is shown for comparison, $D_0 = 11.9(2) \times 10^{-7}$ cm²/s (full gray circle).¹² The value of D_0 for the phosphate buffer was $11.3(2) \times 10^{-7}$ cm²/s, and for the Tris-Gly buffer, it was $11.7(3) \times 10^{-7}$ cm²/s.

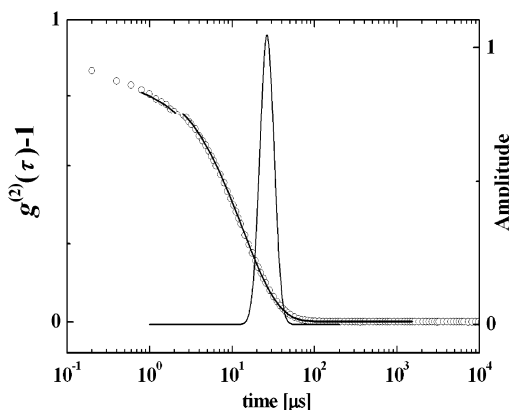


Figure 3. Concentration-concentration autocorrelation function for the lysozyme charge ladder in a Tris buffer (eq 16) as a function of time for the right angle scattering geometry ($\theta = 90^\circ$, $q = [4\pi n_R \sin(\theta/2)]/\lambda$) obtained from the photon correlation spectroscopy experiment (empty circles). It was fitted by a single-exponential function, $\exp(-\tau/\tau_{\text{PCS}})$, (solid line) with $\tau_{\text{PCS}} = 27$ μ s indicating that all proteins in a charge ladder are characterized by the same diffusion coefficient $D = 1/q^2\tau_{\text{PCS}}$. Additionally, the data were analyzed using the power spectrum of possible times using the Conti simulation program available together with a correlator for the PCS (solid line sharply peaked at 27 μ s).

the data. The measured diffusion coefficient is $D = 1.13 \times 10^{-6}$ cm²/s, and from the Einstein-Stokes relation

$$D = \frac{kT}{6\pi\eta R} \quad (17)$$

we determine the hydrodynamic radius $R = 1.9$ nm (the buffer viscosity was determined independently using a Cannon viscometer and a Parr densitometer).

Viscosity Experienced by Lysozyme in the Surfactant Solution

In FCS, we measured the time (residence time) which a lysozyme spends in the focal volume.³⁸ In order to reduce the

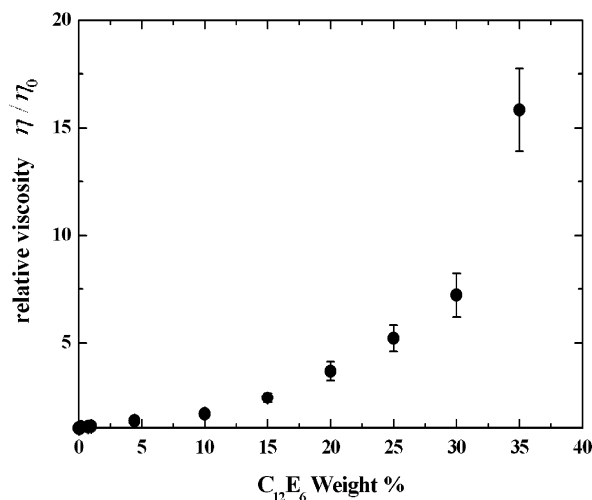


Figure 4. Relative (with respect to buffer) viscosity, η/η_0 , in C₁₂E₆ solutions as a function of concentration, derived from the diffusion coefficient obtained using fluorescence correlation spectroscopy (FCS). In the range of concentrations from 0% to 35% w/w, it can be fitted with an exponential function.

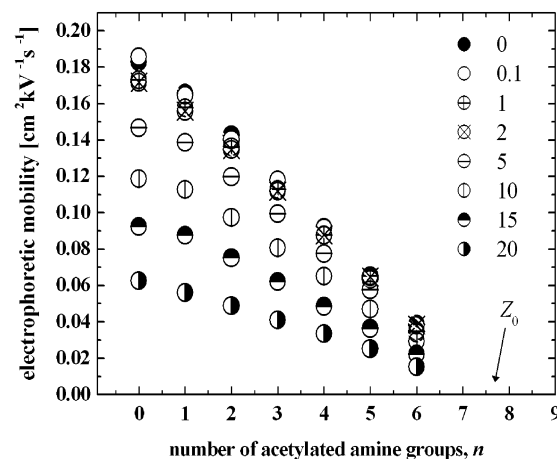


Figure 5. Electrophoretic mobilities, $m^{(n)}$, of the rungs of the lysozyme charge ladder presented for different surfactant concentrations (ranging from 0% to 20% by weight, as indicated in the legend). The slope of the $\mu^{(n)}$ vs n decreases with increasing surfactant concentration, reflecting the increase of the viscosity of the fluid (Figure 4). The charge of the native lysozyme, Z_0 , remains constant for different surfactant concentrations (see also Figure 1).

potential systematic error, we have utilized the following formula that relates the diffusion coefficient, the viscosities, and the average residence times in two different experiments:

$$\frac{D_0}{D} = \frac{\eta}{\eta_0} = \frac{\tau}{\tau_0} \quad (18)$$

where τ is the resident time of the protein in surfactant solution. Here, τ_0 is the resident time of the protein in buffer solution (without addition of the surfactant). D is the diffusion coefficient of the probe in surfactant solution. D_0 is the diffusion coefficient of the probe in buffer solution (without addition of the surfactant). In Figure 4, we show the viscosity as a function of surfactant concentration for lysozyme. The measured microviscosity increases exponentially with the concentration of surfactant. We used the results for viscosity in the analysis of the electrophoretic measurements.

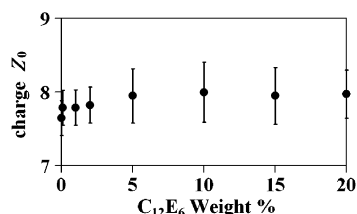


Figure 6. Charge Z_0 (obtained from Figure 5) shown as a function of surfactant concentration. The charge is constant over the range of the studied concentrations. The average value, $Z_0 \sim 7.9 \pm 0.2$, corresponds to the net charge of $+6.1(4)$ measured in units of elementary charge.

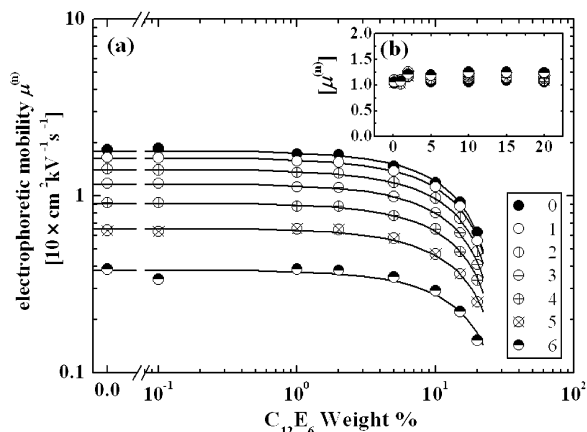


Figure 7. (a) Electrophoretic mobility, $\mu^{(n)}$, for the lysozyme charge ladder $n = 0, 1, 2, 3, 4, 5$, and 6 as a function of surfactant concentration. (b) Normalized mobility $[\mu^{(n)}] = \mu^{(n)}\eta/\mu_0^{(n)}\eta_0$ as a function of surfactant concentration. The normalized mobility is the mobility multiplied by the viscosity determined in FCS measurement (see Figure 4) and divided by the mobility in a pure buffer for every rung n of the lysozyme charge ladder. The term $[\mu^{(n)}]$ is the ratio of the charge of the lysozyme in surfactant solution to the charge in a buffer. The inset provides clear evidence that the protein charge does not change in $C_{12}E_6$ solution; all data follow a single master curve independent from the concentration of surfactant and $[\mu^{(n)}] = 1$ indicating the same charge in a solution and in a buffer.

Electrophoretic Mobility of a Charged Ladder in Surfactant Solution

We present the results obtained for the lysozyme charge ladders in the $C_{12}E_6$ solutions in Figure 5. First, we plot the electrophoretic mobility as a function of the number n of acetylated groups (see also Figure 1) for a range of concentrations of surfactant. The change of slope a reflects the increase of viscosity of the solution (Figure 4) following the increase of the concentration of surfactant in the buffer from 0.1 to 20% (w/w). We notice that—within the experimental error—all the lines intercept the x -axis at the same point indicating that the value of the net charge on the unmodified lysozyme (Z_0) does not change as a function of the surfactant concentration. In Figure 6, we plot Z_0 as a function of the surfactant concentration and conclude that within experimental error (mainly in pH and in the arrival times in the highly concentrated solutions of surfactant) it does not depend on surfactant concentration. In Figure 7a, we show the mobility as a function of surfactant concentration for all rungs. Very large errors in the arrival times for the highest concentration of surfactant (30% w/w) precluded our analysis, thus the highest concentration for which we obtained reasonable results was 20%. Within the experimental error, the electrophoretic mobility multiplied by the viscosity of the solution is constant and independent of the concentration of surfactant (Figure 7b). This observation indicates that nonionic surfactant changes neither the net charge nor the

effective hydrodynamic radius of the lysozyme for every lysozyme derivative in a charge ladder.

Conclusions

Combination of measurements of the electrophoretic mobility and independent measurements of hydrodynamic radii and of viscosity allows for a complete analysis of protein net charge in a model crowded environment. It is especially important to use charge ladders¹³ as they reduce possible sources of errors and allow in a single measurement to determine the number of charge groups on a protein, mobility of an unmodified protein, and the ratio of the charge and the viscosity coefficient. Fluorescence correlation spectroscopy can be efficiently used to determine the viscosity experienced by proteins during Brownian motion in a crowded environment. Photon correlation spectroscopy provides the value of the hydrodynamic radius. Three techniques together allow for determination of any changes in the protein charge or structure upon an introduction into the crowded environment.

We used all three techniques and demonstrated that lysozyme does not change its charge or conformation in the system of nonionic surfactant $C_{12}E_6$. Although at first sight, the result seems to be obvious we have to recall that the chemical equilibrium which determines the protein charge in the buffer depends locally on the counterion cloud around the protein. Originally, we expected that when the environment is crowded the counterion cloud would deform during the motion of a protein when it passed through the narrow gaps between micelles. This in turn would affect the charge on a protein. We found that it is not the case and that the crowded environment does not change appreciably the counterion cloud around the protein and consequently does not change its charge. We expect that similar observations should hold for other nonionic surfactants.

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