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Research Article

Analysis of the interplay among charge, hydration and shape of proteins through the modeling of their CZE mobility data

Electrophorectic mobility data of four proteins are analyzed and interpreted through a physicochemical CZE model, which provides estimates of quantities like equivalent hydrodynamic radius (size), effective charge number, shape orientation factor, hydration, actual p*K* values of ionizing groups, and pH near molecule, among others. Protein friction coefficients are simulated through the creeping flow theory of prolate spheroidal particles. The modeling of the effective electrophoretic mobility of proteins requires consideration of hydrodynamic size and shape coupled to hydration and effective charge. The model proposed predicts native protein hydrations within the range of values obtained experimentally from other techniques. Therefore, this model provides consistently other physicochemical properties such as average friction and diffusion coefficients and packing fractal dimension. As the pH varies from native conditions to those that are denaturing the protein, hydration and packing fractal dimension change substantially. Needs for further research are also discussed and proposed.

Keywords:
Effective electrical charge / Hydration / Hydrodynamic shape / Hydrodynamic size / Protein electrophoretic mobility DOI 10.1002/elps.200800743

1 Introduction

CZE is an effective methodology for the separation and characterization of proteins, peptides, amino acids and, in general, many electrically charged analytes [1]. Innovations involving both protocol formulations and new experimental setups are continuously proposed in the literature. In particular, relevant reviews including CZE of proteins and peptides are available (see, for instance, [2–5] and citations therein). At present CZE models are useful in the physicochemical characterization and interpretation of the effective mobility data of peptides and proteins [6–16]. One model type of interest is that considering the ''inverse problem'' [17] where, for a given protocol involving well-specified bulk pH, ionic strength I, temperature T, electrical permittivity ε and viscosity η of the BGE, the experimental effective mobility is provided as the basic data to evaluate analyte properties such as, for instance, hydration, effective electrical charge, hydrodynamic size and shape, and pH-microenvironment, among others. This basic problem has associated the charge regulation phenomenon, also designated proton-binding cooperativity, which is always present around electrophoretically migrating particles [15–24]. This phenomenon is relevant to estimate the protein p*K*-shifts of the i-ionizing group designated ΔpK_i, the pH near molecule pH*, and the pH near the *i*-ionizing group indicated as pH_i, for $i = 1 \ldots N_c$. Here N_c is the number of ionizing groups in the analyte by accounting also different positions that each one of them occupies in the macromolecular chain, both as amino acid residual and terminal amino and carboxylic groups. Thus, from one pH another, one expects to find deviations of the values taken by acid dissociation constants of ionizing groups in proteins and peptides, and hence the estimation of p*K*-shifts are required [15, 22–30]. In particular, the evaluation and prediction of p*K*-shifts of ionizing groups of amino acids residues in proteins have gained much attention in order to understand better complex biological systems.

In this context of analysis of CZE, this work is concerned with the characterization of four proteins: lysozyme (LSZ), staphylococcal nuclease (STN), human carbonic anhydrase (HCA), bovine carbonic anhydrase (BCA). Their experimental effective mobilities are reconsidered and discussed in relation to a previous work [15] where these proteins were studied as spherical hydrodynamic particles.

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Abbreviations: AAS, amino acid sequence; **BCA,** bovine carbonic anhydrase; **ESM,** equivalent spherical model; **HCA,** human carbonic anhydrase; **LSZ,** lysozyme; **PDB,** protein data bank; **PLLCEM,** perturbed Linderstrøm–Lang CE model; **STN,** staphylococcal nuclease

Also it should be pointed out here that we have analyzed the characterization of proteins, peptides and amino acids through the model designated ''Perturbed Linderstrøm–Lang CE Model'' (PLLCEM) where an equation for the estimation of hydration was also included. Since this model was enhanced in a sequence of works [15–17, 22, 31] as a consequence of the analysis of emerging numerical results and conclusions, the purpose of this particular work is to apply an improved version of the PLLCEM to the characterization of proteins. New inclusions into this model are explicit friction coefficient expressions for prolate spheroidal particles and a relation between hydration and electrical permittivity in the protein domain, in order to discuss and illustrate important aspects concerning the interplay among shape, hydration and effective charge of proteins. Also, in the context of the PLLCEM, part of our results consists in calculating hydrodynamic sizes and shapes of proteins, at each pH and *I*, studied by associating their volumes (hydration is included) with the equivalent hydrodynamic volumes of prolate spheroidal particles.

This work is organized as follows. Section 2 presents briefly the PLLCEM for proteins. The model is solved numerically by studying four proteins, for which CZE-effective mobilities and full protocol data are available in [15]. Also, emphasis is placed on the mathematical strategy to solve the resulting well-posed problem, by identifying the hydration number, estimated at a given pH, and the associated particle shape, through a consistent and convergent computational procedure presented in [15, 17, 22]. Then Section 3 analyses and discusses numerical results of the PLLCEM providing relevant physicochemical properties of the four proteins studied here. Also some limitations associated with this model are pointed out. These considerations indicate challenging requirements for further research on this subject. Finally, Section 4 is dedicated to place the main conclusions of this work.

2 The PLLCEM for proteins

In our previous calculations with the PLLCEM concerning peptides and amino acids, we found that the hydration δ (mass of water *per* mass of analyte) of these particles, at well-specified physicochemical conditions, depended significantly on particle shape and electrostatic state defined through the effective *Z*, positive Z_+ and negative Z_- charge numbers. Here, in the PLLCEM the equivalent spherical model (ESM) of CZE is used again, which has been illustrated in [16, 17, 22] and validated in particular for spheroidal particles of low eccentricity, usually selected as prototype hydrodynamic shapes for proteins and peptides. The ESM has also been used widely in the literature and in basic texts to estimate diffusion and sedimentation coefficients [32, 33]. Basically, the PLLCEM considering the ESM involves the most relevant physicochemical variables of CZE that are fully coupled. To visualize this aspect neatly, this model is described briefly below, by placing the equations required to obtain a consistent solution. The protein amino acid sequence (AAS) is acquired from the Protein Data Bank (PDB), and the PLLCEM processes the main file data by extracting each amino acid position along the main heterogeneous chain to evaluate relative distances r_{ij} between *i*- and *j*-ionizing groups [15]. Therefore, the effective electrical charge *eZ* of the protein is $eZ = \sum_{i=1}^{N_c} eZ_i$, where *e* is the elementary charge and Z_i is the charge number of each ionizing group expressed $Z_i = \pm 1/\{1 + 10^{\mp(\mathrm{p}K_i^{\mathrm{r}} - \mathrm{pH}_i \pm \mathrm{p}\gamma_i)}\}$; here signs are defined according to basic or acid properties of ionizing groups. Also activity coefficients γ_i are included as indicated in [17]. The evaluation of protein-effective charge requires the knowledge of actual $\mathrm{p}K_i$ values of ionizing groups of side chains and ionizing terminal –COOH and $-NH_2$ groups [15, 16, 22, 28]. The $\mathrm{p}K_i$ values are mainly a result of electrostatic interactions among protein ionizing groups and ions in the BGE, yielding a shift $\Delta\mathrm{p}K_i$ of the reference $\mathrm{p}K_i^{\mathrm{r}}$, which in turn refers to the hypothetical p*K* value where one assumes that all other titrating sites in the analyte are fixed in their electrically neutral state (see [22] for a discussion concerning $\mathrm{p}K_i^{\mathrm{r}}$ and also Section 3). Thus, the pH-microenvironment around the *i*-ionizing group, designated pH_i, is a function of physicochemical properties of the electrolyte solution surrounding the macromolecule, and also of the protein structural parameters as described below. It may be expressed [15],

$$\mathrm{pH}_i = \mathrm{pH}^* + \frac{e^2}{\ln(10)k_{\mathrm{B}}T} \times \left(\frac{\Delta Z_i}{4\pi r_i^{\mathrm{o}}\varepsilon}\left(\frac{\varepsilon}{\varepsilon'} - 1\right) + \sum_{\substack{j=1 \\ j\neq i}}^{\mathrm{Nc}} \frac{\Delta Z_j}{4\pi\varepsilon'} \frac{\exp(-\kappa' r_{ij})}{r_{ij}} \right) \quad (1)$$

where the Debye and Hückel's approximation and Henry's hypotheses are invoked, and the screened Coulombic interactions among ionizing groups are included. In Equation (1), $\Delta Z_j = Z_j - Z_j^{\mathrm{r}}$, where Z_j^{r} is the reference charge number at site *j* calculated with $\mathrm{p}K_j^{\mathrm{r}}$. Also, Z_j is the actual charge number at site *j*, $r_i^{\mathrm{o}} \approx 1.4$ Å is the approximate effective radius of the *i*-ionizing group, k_{B} is Boltzmann constant and ε′ is the electrical permittivity within the protein domain. Since the inverse of the screening length is $\kappa = \sqrt{2e^2 N_{\mathrm{A}} \mathrm{I} 10^3/\varepsilon k_{\mathrm{B}} T}$, where N_{A} is Avogadro constant, one obtains $\kappa' = \kappa\sqrt{\varepsilon'/\varepsilon}$. The estimation of ε′ is a difficult problem already placed in the literature [15, 34]. In this work, we found appropriate to estimate, as a first approximation, a fractional weight averaged electrical permittivity within the domain of the protein through $\varepsilon' = \varepsilon\delta/(1+\delta) + \varepsilon_{\mathrm{p}}/(1+\delta)$, where $\varepsilon_{\mathrm{p}} \approx 5\ \varepsilon_{\mathrm{o}}$ is an approximate value of the dry protein electrical permittivity [34] and δ is the convergent value coming from the numerical iteration process described elsewhere [17]. Also ε_{o} is the vacuum electrical permittivity. In Equation (1), pH* is evaluated by using the mean field approximation expressed [15],

$$\mathrm{pH}^* = \mathrm{pH} + \frac{e^2 Z}{\ln(10)k_{\mathrm{B}}T 4\pi\varepsilon a_{\mathrm{H}}^{\mathrm{e}}(1 + \kappa a_{\mathrm{H}}^{\mathrm{e}})} \quad (2)$$

In Equation (2) the protein particle has a surface potential [35] considered in the particle-effective mobility within the context of the ESM. Thus, the relevant variable is the equivalent

sphere with a Stokes equivalent hydrodynamic radius a_H^e, which must be defined in terms of the main particle dimensions, as shown below. Specific discussions concerning the physical significance of each term in Equations (1) and (2), may be found elsewhere [15, 16, 22]. For calculation purposes, the expression $\Delta pK_i = pK_i^r - pK_i = pH_i - pH = \Delta pH_i$ is still valid here (see [15] and its addendum for a detailed discussion on this expression). Therefore, ΔpK_i of ionizing groups are also a direct consequence of pH shifts designated ΔpH_i, which are the differences between the pH near ionizing groups in the protein and the pH of the BGE. Thus, Z_i expressed above in terms of $(pK_i^r - pH_i)$, where pH_i is the unknown to be determined, may be also calculated through $(pK_i - pH)$ by using the bulk pH, where alternatively the unknown is pK_i.

The model is completed by considering that the protein electrophoretic mobility may be expressed as follows:

$$\mu_p = \Omega\mu \tag{3}$$

where

$$\mu = eZf(\kappa a_H^e)/\{6\pi\eta a_H^e(1+\kappa a_H^e)\} \tag{4}$$

In Equation (3), Ω is the shape orientation factor [16] and μ is the effective mobility of the equivalent spherical particle defining the ESM. Also $f(\kappa a_H^e)$ is Henry's function reported in [36]. Then it is clear that the equivalent hydrodynamic radius a_H^e and the shape of the migrating particle are necessary in order to define properly the average particle friction coefficient f, when the continuum mean field hypothesis is invoked, as it is analyzed below. Therefore, several important considerations must be carried out in relation to the effective protein mobility. First, one should observe that the size of proteins is within the scale range of Brownian particles and, hence, they move in the direction of the applied electrical strength vector with a random orientation. Thus, for particles with rotational symmetry considered here, Ω may be obtained from the frictional tensor $\underline{\underline{f}}$ [37, 38] expressed through body Cartesian coordinates with unit vectors $\underline{i}$, $\underline{j}$ and $\underline{k}$, as follows:

$$\underline{\underline{f}} = f''\underline{k}\underline{k} + f^{\perp}(\underline{i}\underline{i} + \underline{j}\underline{j}) \tag{5}$$

where f'' is the friction coefficient when the particle movement is parallel to the rotational axis of symmetry, while $f^{\perp}$ is the friction coefficient perpendicular to this axis for any rotational angle. Therefore, the average friction coefficient f of a rotational symmetric particle is expressed,

$$\frac{1}{f} = \frac{\alpha}{f''} + \frac{(1-\alpha)}{f^{\perp}} \tag{6}$$

where $0<\alpha<1$ indicates the degree of the collinear particle orientation with the applied electrical field direction, as described in [16, 17, 22]. Thus, α provides the fractional weight that friction coefficients f'' and $f^{\perp}$ have on f. This result also implies the existence of an orientation density distribution function as discussed in Section 3 below (see also [39]). From the above equations, one readily verifies that Ω satisfies the following expression:

$$\Omega = \frac{6\pi\eta a_H^e}{f} = \frac{f_0}{f} \tag{7}$$

Thus, Equation (7) indicates that Ω is equivalent to the inverse ratio f/f_0 classically used in the literature [32]. After solving the PLLCEM, the second step consists in attributing a given shape to the particle by having into account numerical values Ω and a_H^e. For this purpose, the components of tensor $\underline{\underline{f}}$ for prolate spheroids may be expressed in terms of the major radius a, which in this case is placed along the rotational axis of symmetry, and the eccentricity $E_x = \sqrt{1-(c/a)^2}$, as follows:

$$f'' = \frac{8\pi\eta a E_x^3}{(1+E_x^2)\frac{1}{2}\ln\left\{\frac{1+E_x}{1-E_x}\right\} - E_x},$$
$$f^{\perp} = \frac{16\pi\eta a E_x^3}{(3E_x^2-1)\frac{1}{2}\ln\left\{\frac{1+E_x}{1-E_x}\right\} + E_x} \tag{8}$$

where c is the prolate minor radius. Expressions for oblate spheroids may also be obtained from the literature [38, 40]. Here it should be observed that the ESM involves the following aspects to be accounted carefully: (A) the surface potential $\zeta = eZ/\{4\pi\varepsilon a_H^e(1+\kappa a_H^e)\}$ of the equivalent sphere is the same as the particle surface potential expressed in terms of a relation $a_H^e = a_H^e(a, E_x)$ derived from this constraint. This relation for a prolate spheroid is $a_H^e \cong 2aE_x/\ln\{(1+E_x)/(1-E_x)\}$ [16] when $\kappa a E_x < 1$ [41] and $aeE_0/(k_BT) < 1$ [35], where E_0 is the applied electrical field strength. For this purpose, the asymptotic expression of the particle surface potential provided in [41] for the prolate spheroid is useful; in this work a mobility expression is also reported when this particle is weakly charged and ion relaxation is not included (see also the discussion below, at the end of Section 3). (B) The volume of the particle must equal the volume of the equivalent sphere; thus $a_H^e = a \times (1-E_x^2)^{1/3}$ for prolate spheroids. Conditions (A) and (B) are asymptotically satisfied for $E_x < 1$ (even for values near one, as it may be readily proved numerically).

Once values Ω and a_H^e are known when convergence criteria described in [22] of the numerical code [15] are satisfied, the resulting hydrodynamic particle shape and orientation may be fixed by considering that prolate spheroidal particles migrate with an average friction coefficient described by Equations (6) and (8). Further, when all the prolate particle orientations are equally probable for the Eulerian angles (a random flight orientation density distribution function is assumed) one gets $\alpha = \frac{1}{3}$ [37]. Nevertheless, charged analytes have an electrostatic interaction with E_0 having a tendency to bias pure Brownian motion. This is one of the reasons for obtaining values $\alpha \neq \frac{1}{3}$ in CZE calculations [16]. For rotational symmetric particles, the angle θ formed between the prolate axis of revolution and the direction of the electrical field E_0 may be estimated from α. Thus, $\alpha = \cos^2\theta$ and $1-\alpha = \sin^2\theta$ on the average [17], and hence $\theta \approx 55^\circ$ for $\alpha = \frac{1}{3}$. As a consequence of this result, when $\theta > 55^\circ$ or $\alpha < 1/3$, the friction tensor component that predominate in the particle migration is $f^{\perp}$ for prolate spheroids. The opposite is also true for $\theta < 55^\circ$ and $\alpha > \frac{1}{3}$ involving mainly f''. It is then clear that the calculations

concerning approximate main dimensions a and c of prolate spheroids and their average orientation in relation to the direction of the electrical field strength may be estimated through Equations (6)–(8), although they are not required here for discussing most of numerical results in Section 4, because any generic particle representing the protein hydrodynamics is already determined through parameters Ω and a_H^e [22]. Thus, since this reasoning can be extended to other particle shapes apart from spheroids, Ω and a_H^e are quite general hydrodynamic parameters for proteins within the context of Debye and Hückel's approximation and Henry's hypotheses applied to CZE.

The closure of the PLLCEM requires the introduction of the hydration number H (number of water molecules *per* analyte molecule) of the migrating protein, as a relevant property associated with the particle shape. Based on the ESM, the protein hydrodynamic volume $V_H = 4\pi a_H^{e^3}/3$ includes the protein compact volume $V_c = M\nu_p/N_A = 4\pi a_c^3/3$ with a compact radius a_c. Here M is the protein molar mass and ν_p is the protein specific volume calculated through $\nu_p = \sum_{i=1}^{N} M_i\nu_i/M$, where ν_i is the specific volume [33] and M_i is the molar mass of each amino acid residue composing the protein AAS as obtained from the PDB. The volume $(V_H - V_c)$ is due to hydration, indicating that $a_H^e > a_c$. Therefore, an estimate of δ can be obtained from the numerical knowledge of both a_H^e and a_c (expressed in $\text{Å} = 10^{-10}\,\text{m}$) through $\delta \approx [(a_H^e/a_c)^3 - 1](\nu_p/\nu_w)$ once the model solution provides a_H^e. Also $\nu_w \approx 1\,\text{cm}^3/\text{g}$ is the specific volume of the BGE. In addition for the calculations carried out in this work, an estimation of protein hydration number $H = \delta M/18$ is obtained by summing each hydration number reported in the literature of polar and ionizing, polar and non polar groups as follows [22]:

$$H \approx h^{I}_{t-COOH}|Z_{t-COOH}| + h^{I}_{t-NH_3^+}|Z_{t-NH_3^+}| + h^{PI}_{t-COOH}(1 - |Z_{t-COOH}|) + h^{PI}_{t-NH_3^+}(1 - |Z_{t-NH_3^+}|) + \sum_{i}^{N_c}\{h_i^I|Z_i| + h_i^{PI}(1 - |Z_i|)\} + \sum_{i}^{N_p} h_i^P + \sum_{i}^{N_{np}} h_i^{NP} \quad (9)$$

where subscripts t and i refer to terminal groups and side chains of residual amino acids, respectively, and N_p and N_{np} indicate the number of polar and non-polar amino acid residues composing the protein. It is clear that Equation (9) applies mainly for the native states of proteins as a first approximation, where most of the protein water is associated with ionizing h_i^I and polar h_i^{PI}, polar h_i^P and non-polar h_i^{NP} amino acid residue hydration numbers, as described previously for amino acids, and more roughly for peptides [17, 22]. In this work, approximate values of hydration numbers of amino acids residuals provided by Kuntz [42] are used and also estimations carried out in [22] are included. It is clear that the value of δ calculated with the PLLCEM applied to native proteins must be consistent with the hydration number H estimated with Equation (9). Concerning the hydration of proteins, one should observe that in classical hydrodynamic models, the estimation of f/f_0 has been a difficult task because in general δ is undetermined. Thus approximate hydration values from other techniques were required even when protocol tests are not necessarily equivalent. Alternative analyses considered, for instance, the maximum ratio $(f/f_0)_{max}$ corresponding to a spherical shape [32]. Here instead we use an estimation of δ, which is a result of the PLLCEM, consistent with Equation (9), where the electrostatic state and particle shape are fully coupled to hydration [22]. For protein denatured states Equation (9) must be modified because, under these circumstances, proteins may gain or eventually loose structural water due to additional solvent-particle or intra-particle interactions [43] not involving necessarily the considerations stated above to obtain Equation (9). Thus, an effective hydration number $H_{eff} = H + H_d$ for protein denatured states is required, where H_d includes the effect of protein water evolution from the native to unfolded states. Certainly, at present the term H_d is still difficult to estimate (see also the discussion below concerning the study of STN at different pH).

Once the main physicochemical properties described above have been evaluated for a given experimental mobility data through the PLLCEM, the packing fractal dimension of the protein may be estimated (see, for instance, definitions in [35] for a generic chain). In this sense it is known that water molecules control substantially the structure, stability and function of biomolecules [44, 45] and that hydration forces are responsible for the packing and stabilization of protein structures by participating mainly in hydrogen bonds. Therefore, the protein packing within the hydrodynamic volume defined through a_H^e requires the consideration of the number N of total amino acid residues with an average monomer radius $a_0 = \sum_{i=1}^{N} a_i/N$ (different monomers forming the heterochain), where each amino acid residue has a radius $a_i = \{3\nu_i M_i/(4\pi N_A)\}^3$. Therefore, the volume fraction of amino acids composing the protein in the hydrodynamic volume is obtained by observing that one unit of effective radius a_H^e is equal to N units of average radius a_0 with packing fractal dimension, $g = \log N/\log(a_H^e/a_0) \leq 3$. It is then clear that for $g = 3$ the compact protein volume is obtained with $a_H^e = a_c$ and $\delta = 0$. It should be placed emphasis here on that a_H^e is calculated through the PLLCEM and hence this hydrodynamic radius provides the full spatial sweeping of the protein, where other constraints apart from hydration are implicitly involved, like electrostatic interactions, binding ions, number of disulfide bridges, *etc.* Thus, a_H^e and g carry a condensed structural information of the protein in some degree. In addition, other relevant protein properties of practical interest like average friction coefficient $f = 6\pi\eta a_H^e/\Omega$, Stokes–Einstein diffusion coefficient $D = k_B T\Omega/(6\pi\eta a_H^e)$ and sedimentation coefficient $s = \Omega M(1 - \nu_p/\nu_w)/\{6\pi\eta a_H^e N_A\}$ may be evaluated with numerical data provided by the PLLCEM at the running protocol conditions.

3 Results and discussion

The present version of the PLLCEM described in Section 2 was run for the four proteins studied with CZE protocols and physicochemical conditions already described in [15], where data required with the corresponding references were reported. Here the set of reference pK_i^r provided in [28] is used. Therefore, Table 1 shows numerical results at 25°C for STN [46], HCA [47], BCA [20] and LSZ [23], which are considered to be in their native states. In this table one observes that hydration values ($0.35 < \delta < 0.45$) obtained through the PLLCEM are similar to those reported previously within the range 0.2–0.5, when X-ray diffraction, NMR, spectroscopy and molecular dynamics simulations were used (see, for instance, citations in [48, 49]). These results indicate that Equation (9), which is coupled to basic equations of the PLLCEM through electrical charge numbers, is a good estimate of native protein hydrations. Further, it is observed consistently that when the protein hydration increases, the required ε' for an appropriate numerical convergence of the PLLCEM with the expression $\varepsilon' = \varepsilon\delta/(1+\delta) + \varepsilon_p/(1+\delta)$ becomes higher as expected, having ε of the BGE as limit value (see also below this physical aspect in relation to the denatured state of STN). For the four native proteins $20 < \varepsilon'/\varepsilon_0 < 30$, as it is reported in Table 1. It is interesting to place emphasis on that the PLLCEM is able to provide an estimate of the electrical permittivity within the protein domain. In this sense preliminary values of ε' may be useful for further numerical researches in more involved molecular dynamic calculations, having into account that this is an unsolved task already placed in the literature (see also the discussion in [34]). In addition, Table 1 presents values of pH* indicating that the difference between pH and pH* may be as high as 0.68 units for these proteins and protocols, showing clearly the importance of considering the charge regulation phenomenon through either ΔpK_i and ΔpH_i, where the effective, positive and negative charge numbers reported in this table are quantitatively required for the structural and functional understanding of globular proteins.

Another relevant result from the PLLCEM is that $\Omega < 1$ (Table 1) and hence $f/f_0 > 1$ for the four proteins studied here (Table 2). Further, as f/f_0 increases (Ω decreases) these proteins have a tendency to be less hydrated for $\Omega < 1$ (δ becomes relatively smaller) although there are other factors affecting the final result of hydration due to the nonlinear coupling of physicochemical variables, as it is indicated in Section 2 by the full set of equations. Nevertheless, the proteins studied here present hydrodynamic particle shapes closer to the spherical one as the values of hydration become higher. Also numerical results of f/f_0 obtained with the PLLCEM are in good agreement with the values reported through other estimations [32, 33]. From Table 2, important conclusions may be obtained from the comparisons of hydration values when $\Omega = 1$ (spherical shape) and $\Omega < 1$ (deformed sphere). Thus, the hydrations of these hydrodynamic particles are not necessarily the same, and hence results demonstrate the complex interplay among shape, effective charge and hydration of proteins, having into account that for $\Omega = 1$ [15] it is not possible to use Equation (9) as a constraint in the PLLCEM for the calculation of H (over specified number of equations), and hence the hydration δ obtained only from a_H^e and a_c is considered to be the maximum value as indicated elsewhere [32]. Further, Table 2 also reports physicochemical properties of practical interest such as protein friction, diffusion and sedimentation coefficients, and average protein specific volume as obtained directly from the numerical output of the PLLCEM. These results are in good agreement with previous values reported in the literature for similar conditions, obtained either theoretically as well as experimentally (see, for instance, [33, 48, 50, 51]). In principle, one finds a remarkably consistency among predictions of protein friction coefficients reported in the literature, having into account that this parameter is common to most of hydrodynamic types of experimental techniques. Nevertheless, the major question arrives when one needs to interpret the meaning of the numerical average friction coefficient of Brownian particles in terms of the components of the friction tensor (Equations (6) and (8)) involving the nature of the orientation density distribution function during particle movement. In fact, in the evaluation of the sedimentation coefficient [52], the effect of electrical charges in analytes is not analyzed neither required and, hence, the average friction coefficient may be interpreted simply through Perrin factor calculated with $\alpha = \frac{1}{3}$ in Equation (6) [32, 40]. As pointed it out above, this is not precisely the case of proteins moving in CZE where the interaction between particle and

Table 1. Numerical results of electrical permittivity within protein domain ε', shape orientation factor Ω, equivalent hydrodynamic radius a_H^e, effective Z, positive Z_+ and negative Z_- charge numbers, pH near molecule pH* and hydration δ provided by the PLLCEM for the native proteins studied in this work at 25°C. Ionic strength I and pH of the BGE, and protein molar mass M and electrophoretic mobility μ_p are also included

Protein	M (g/mol)	$\mu_p \times 10^9$ ($m^2 V^{-1} s^{-1}$)	pH	I (mM)	$\varepsilon'/\varepsilon_0$	Ω	a_H^e (Å)	Z	Z_+	Z_-	pH*	δ
STN	16792	18.1	6.8	26	28	0.949	19.70	8.96	29.99	21.03	7.40	0.449
HCA	29100	4.11	8.4	10	26	0.935	23.34	−2.07	30.98	33.05	8.26	0.403
BCA	28982	6.20	8.4	10	26	0.805	23.27	−3.34	28.03	31.37	8.15	0.397
LSZ	14300	16.7	8.4	8	24	0.788	18.09	6.76	16.88	10.12	9.08	0.346

the applied electrical field requires to be accounted, usually giving $\alpha \neq \frac{1}{3}$ (see also Tables 2 and 3 in [16]). The validation of this conclusion follows from Table 3. In fact, the dimensions estimated of native proteins and reported in this table are obtained from Equations (6)–(8) as follows. First, since a_H^e is provided by the PLLCEM output (Table 1), the approximate major radius is adopted with $a > a_H^e$, following typical values reported in the literature, and hence the smaller radius $c < a_H^e$ is calculated from the condition that the protein volume must equal the volume of the hydrodynamic equivalent sphere. Then α is evaluated such that

Table 2. Comparison of hydration values δ between spherical ($\Omega = 1$) and deformed spherical ($\Omega < 1$) particles, and values of friction ratio f/f_0, packing fractal dimension g, friction f, diffusion D and sedimentation s coefficients and average specific volume v_p provided by the PLLCEM output of native proteins studied in this work

Protein	δ ($\Omega = 1$)	δ ($\Omega < 1$)	f/f_0	g	$f \times 10^{11}$ (kg/s)	$D \times 10^{11}$ (m^2/s)	$s \times 10^{13}$ (s)	v_p (cm^3/g)
STN	0.460	0.449	1.02	2.73	3.53	11.65	2.22	0.719
HCA	0.570	0.403	1.07	2.77	4.24	9.70	3.21	0.718
BCA	1.090	0.397	1.24	2.77	4.91	8.37	2.78	0.716
LSZ	1.230	0.346	1.27	2.75	3.90	10.53	1.78	0.707

Table 3. Approximate prolate spheroid dimensions a and c, eccentricity E_x, orientation angle θ and components f'' and $f^{\perp}$ of the friction tensor for native proteins studied in this work, obtained from Eqs. (6) and (8), volume conservation and data of the shape orientation factor Ω and equivalent hydrodynamic radius a_H^e provided by the PLLCEM output

Protein	a_H^e (Å)	Ω	a (Å)	c (Å)	E_x	θ (°)	$f'' \times 10^{11}$ (kg/s)	$f^{\perp} \times 10^{11}$ (kg/s)
STN	19.70	0.949	28	16.5	0.807	73.9	3.21	3.56
HCA	23.34	0.935	34.5	19.2	0.831	79.4	3.79	4.26
BCA	23.27	0.805	54	15.3	0.959	80.3	3.92	4.95
LSZ	18.09	0.788	45	11.5	0.967	76.7	3.09	3.96

the numerical value Ω (Table 1) obtained with the PLLCEM is fitted. It is thus found that the angle between the major prolate radius and the direction of the applied electrical field strength is within the range $70 < \theta < 80°$. These approximate values of θ are consistent with the fact that $f/f_0 > 1$ when $\theta > 55°$ providing thus relatively high average particle friction for this type of hydrodynamic particles (see at the end of this section and also [16, 17, 22] for $f/f_0 < 1$ and $\theta < 55°$ involving some amino acids and peptides). A possible explanation of these results should be found on the estimation of the interaction energy between the effective dipole subject to Brownian motion and the electrical field [53], indicating that for small dipole moments, in general of the order of $2a_H^e eZ$ for proteins, the dipole axis forms an angle φ with the direction of the electrical field strength that is rather closer to $\pi/2$ (typically $\{2a_H^e eZ/(3k_B T)\} < 0.1$ for proteins, giving a Langevin function approximately equal to its independent variable). Therefore, as long as the effective dipole axis has a direction close to that of the prolate axis of revolution ($\varphi \approx \theta$) one may find these arguments as a viable explanation concerning the range of values calculated for θ. Further, one should observe here that the analysis of angle φ is carried out through the use of Boltzmann energy distribution function, which not necessarily involves the orientation random flight distribution used to determine $\alpha = \frac{1}{3}$ for particle sedimentation. Table 3 provides values of the friction tensor components f'' and $f^{\perp}$ of the four native proteins validating the relation $f'' < f < f^{\perp}$ and also showing that f is closer to $f^{\perp}$ as a consequence of the relatively high values of θ obtained as explained above.

Numerical results provided by the PLLCEM in Table 1 are used to evaluate g in a given BGE. Thus, for the native proteins studied here a rather constant packing fractal dimension is found as reported in Table 2, comprised in the range $2.73 < g < 2.77$. This minute range of g is consistent with the small hydration range found in the literature for native proteins. Thus a fractal dimension, defined by considering both geometric scales of the amino acid residuals and the equivalent hydrodynamic radius of the ESM, is appropriate to understand the spatial sweep of the protein with its associated water molecules as deduced from CZE in a well specified BGE. More interesting is to observe in Table 4 that for the denatured STN this fractal dimension decreases significantly indicating a rather open structure, where additional water molecules are present and hence $H_d \neq 0$. In fact, by starting from pH 6.8 as native state (see Table 1) one observes in Table 4 that for decreasing pH, the evolution of ε' allowing a convergence of the numerical code, changes toward higher values (from 28 ε_0 to 75 ε_0), which is consistent with the incorporation of further water molecules into the protein (hydration increases). Thus, H_d increases as the pH is lower and the protein becomes denatured (from 0 to 16894 water molecules added). This situation is consistent with the fact that for denature states of proteins, water not only interacts with ionizing, polar and hydrophobic groups but proteins can also be in general invaded by water through rather complex mechanisms involving, for instance, substitution of protein–hydrogen bonds by water–hydrogen bonds, disulfide reorganization, molten hydrophobic core and others phenomena described in [43] and citations therein. When $\Omega < 1$, hydrations values in Table 4 are, as one expects, lower than those calculated with the previous results for the STN, where this protein was assumed to be a spherical particle [15]. Further, these results indicate that hydration values predicted for other denatured proteins reported in [31] correspond to the maximum water invasion where $\Omega = 1$ was imposed. Once more, one may conclude that understanding the interplay among particle shape, hydration and effective charge is essential to characterize proteins in a given BGE. Through the above discussion it is evident that the

Table 4. Numerical results provided by the PLLCEM output for the study of STN at different pH as follows: electrical permittivity within protein domain ε', shape orientation factor Ω, equivalent hydrodynamic radius a_H^e, effective charge number Z, pH near molecule pH*, number of water molecules added H_d, hydration δ and packing fractal dimension g. The other variables reported are pH, ionic strength I and experimental electrophoretic mobility μ_p

pH	I (mM)	$\mu_p \times 10^9$ ($m^2 V^{-1} s^{-1}$)	$\varepsilon'/\varepsilon_0$	Ω	a_H^e (Å)	Z	pH*	H_d	δ	g
6.8	26	18.1	28	0.949	19.70	8.96	7.40	0	0.449	2.73
5.7	36	18.5	43	0.972	22.71	11.32	6.29	570	1.062	2.53
4.1	55	20.4	55	0.889	26.51	20.96	4.79	1577	2.102	2.35
2.8	5.5	24.5	75	0.919	50.34	30.15	3.63	16894	18.485	1.80

analysis of these results also indicates the relevance of parameter ε' in protein characterizations affecting the terms involving Born hydration and screened electrostatic interaction energies of ionizing groups as stated through Equation (1). For this purpose the first approximation constraint $\varepsilon' = \varepsilon\delta/(1+\delta) + \varepsilon_p/(1+\delta)$ proposed here is of course required and appropriate. Nevertheless, it should be pointed out that one may define different levels of protein water invasion by getting consistency with different values of ε' when the denatured state is considered. This is a consequence that within the present framework there is not available an equation estimating the value H_d by starting from the knowledge of protein AAS and protocol conditions, placing thus a clear challenging for future researches on CZE modeling of analyte mobility. Interesting is the fact that approaching the p$I \approx 9.9$ of the STN, the PLLCEM provides, for example at pH 8.9, the following results: $\varepsilon' \approx 20\ \varepsilon_0$, $\delta \approx 0.255$, $H_d \approx -176$ (water molecules are extracted), $a_H^e \approx 18.52$Å and $\Omega \approx 1.5$. This is a reasonable state for the STN at a pH rather close to the pI (the pH near molecule is even higher; *viz.*, pH* = 9.18) having into account that positive and negative charges tend to collapse together, which in turn take less hydration ($H_d < 0$) than that predicted by Equation (9), generating thus a tightly compact structure. Also for these calculations one finds a consistent result concerning the equivalent hydrodynamic radius in the sense that it is lower than the value for pH 6.8. Certainly, this specific and difficult aspect concerning the mobility of the STN at pH close to pI places an additional point for further research concerning the need of an expression to guide the evaluation of H_d, as indicated above. Finally, the value $\Omega > 1$ found here, requiring a relatively low average friction coefficient ($f/f_0 < 1$ and $\theta < 55^\circ$) for the collapsing particle at pH near the pI, is perhaps a consequence that the effective dipole axis has a direction rather normal to that of the prolate axis of revolution with $\theta << \varphi$ (see the counterpart discussion above) although further conclusions on this aspects would require additional research of protein mobility in this high pH range.

Following the discussion of results, it is appropriate to point out here some limitations usually found in simple models like the PLLCEM, mainly for studying particles that are highly charged. Thus, at present it is important to visualize that the PLLCEM has been developed within the framework where the well-known Debye–Hückel's approximation and Henry's hypotheses apply. This means roughly that the valid range of application of this model may be estimated from the reduced mobility $Y = 3\mu_p e\eta/(2\Omega f(\kappa a_H^e)\varepsilon k_B T)$ and surface potential $X = e\zeta/(k_B T)$, and the dimensionless equivalent hydrodynamic radius $P = \kappa a_H^e$ (see also [17]), which were defined in [54] for spherical particles; they are adapted here for the ESM by including Ω and $f(\kappa a_H^e)$. In [54] numerical solutions of the electrophoretic mobility that consider the distortion of the counter-ion cloud of the spherical particle were reported through these parameters. Therefore, it is clear that the PLLCEM is able to provide appropriate results when $Y \cong X$ for $X < 2$ and $P < 3$, $X < 1$ and $P > 3$, or eventually for very low and high values of P, where Hückel and Smoluchowski limits would be reached, respectively, as indicated in [15,17]. In fact, when these constraints are not satisfied, one expects to find the distortion of the counter-ion cloud around the migrating particle causing a reduction in the effective mobility, which may be observed as a nonlinear response of Y *versus* X, passing through markedly maxima when $P > 3$. This phenomenon is known generically as "ion relaxation", and it deserved intensive research in the literature (see, for instance, a discussion on this aspect in [35] and citations therein). In this sense, it is relevant to point out that at present, rigorous studies [55, 56] based on the boundary element method (see, for instance, [38] for a description of the method) are available, which are capable of evaluating the ion relaxation effect with either spherical [55] and arbitrary irregularly shaped particles [56] having a non-centrosymmetric charge distribution; further, the translation–rotation coupling resistance tensor is included. These studies also indicate that for typical proteins with relatively small effective charge (like those proteins studied here) the distortion of the counter-ion cloud is not significant in contraposition with what occurs when short DNA fragments are considered [56]. Our calculations for the four native proteins yield $0.32 < X < 1.57$ and $0.53 < P < 1.04$, while for the STN at different pH, results indicate that $1.35 < X < 1.91$ and $1.04 < P < 2.04$, thus validating our results obtained, in principle, without the consideration of ion relaxation.

In particular, within the range where the PLLCEM applies (ion relaxation may be neglected) another relevant study [57] concerning the slightly deformed sphere [58] with non-centrosymmetric charge distributions is useful to be considered here, in order to visualize the application of the

PLLCEM in this context of analysis. In [57], the linear mathematical structure of the resulting model allows one to obtain the electrophoretic mobility averaged with an orientation density distribution function where all particle orientations are equally probable, through the shape and charge multipole expansions of the particle. Charge multipoles (up to order two) in turns are physically defined as charge monopole (effective charge), dipole and quadrupole, evaluated through the coordinates of charged atoms provided from the PDB (see also [55]). It is found that quadrupoles affect the electrophoretic mobility of a spheroidal particle, which is mainly driven due to its effective charge. Perhaps a question is whether or not all particle orientations are equally possible in CZE. A similar studied was also carried out by Fair and Anderson [39] by using, however, the multipoles required as they resulted from a variable particle surface potential (arbitrarily defined) where spheroidal particles were considered. This particular work places emphasis in that an orientation density distribution function needs to be found before the average electrophoretic mobility can be evaluated, as a consequence of the presence of Brownian motion and the applied electrical field. These results are consistent with the Equation (6) above. Further, in relation to these works [39, 57], it is clear that although the simple formulation of the PLLCEM considers a centrosymmetric charge distribution of the particle (as a consequence of the constraint $\kappa a E_x < 1$, used in the ESM) a correction of the effective charge (monopole) due to Coulombic interactions between pairs of charged atoms is included, which requires also information from the spatially charge distribution within the protein obtained from the PDB (see second term in the right-hand side of Equation (1)) which may be an appropriate estimation of the small effect caused by a non-centrosymmetric charge distribution at this simple level of modeling a rather complex phenomenon. Here one should observe that the exact solution provided in [41] indicates that the maximum value of charge density occurs where the radius of curvature of the particle surface is the minimum (around the tips of the prolate spheroid). Although this effect is lost in the PLLCEM due to the use of the asymptotic expression of this solution for $\kappa a E_x < 1$, the effect is recaptured through the electrostatic perturbations described in Equation (1). Finally the aspect considering the orientation of the prolate spheroid through parameter α in Equation (6) of the PLLCEM seems to be an important condition for weakly charged particles under Brownian motion and an applied electrical field, which is related to the analysis already carried out in [39]. It is perhaps this effect quite relevant in order to obtain consistent numerical values of electrophoretic mobility and diffusion coefficient of relatively low charged proteins with the corresponding experimental data, which is an important subject studied during the last decade in the literature. In this context of analysis the PLLCEM seems to model appropriately the CZE of the four proteins considered in this work, despite its limitations mainly within the nonlinear response of complex phenomenae.

4 Concluding remarks

Estimates of protein hydration ought to be included in CZE models to obtain the appropriate interplay between hydration and shape orientation factor, equivalent hydrodynamic radius and effective charge number of migrating proteins. This result was in part a consequence that hydration predicted through the PLLCEM became very sensitive to the hydrodynamic particle shape and orientation. The importance of simple models like the PLLCEM to visualize physically protein characterizations is demonstrated through the study of four proteins. At present the PLLCEM requires a better interpretation of parameter H_d in terms of the microstructure of the denatured protein, apart from the possibility that more complex shape particle models may be required at this level of details. Also the interaction energy between the effective protein dipole moment and the applied electrical field affecting particle orientation seems to be a subject for further research. Finally, the packing fractal dimension of native and denatured proteins is a relevant characterizing parameter obtained from CZE, which involves hydration as a main factor apart from geometric considerations of protein primary structure. It is also clear that in order to study highly charged analytes, like for instance short DNA fragments, the PLLCEM should be improved by considering irregular-shaped particles and ion relaxation effects.

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5 References

[1] Kenndler, E., in: Khaledi, M. G. (Ed.), *High-Performance Capillary Electrophoresis: Theory, Techniques and Applications*, Wiley, New York 1998, pp. 25–76.

[2] Dolník, V., *Electrophoresis* 2006, *27*, 126–141.

[3] Dolník, V., *Electrophoresis* 2008, *29*, 143–156.

[4] Kašička, V., *Electrophoresis* 2006, *27*, 142–175.

[5] Kašička, V., *Electrophoresis* 2008, *29*, 179–206.

[6] Offord, R. E., *Nature* 1966, *211*, 591–593.

[7] Cifuentes, A., Poppe, H., *Electrophoresis* 1995, *16*, 516–524.

[8] Cross, R. F., Wong, M. G., *Chromatographia* 2001, *53*, 431–436.

[9] Cross, R. F., Garnham, N. F., *Chromatographia* 2001, *54*, 639–646.

[10] Adamson, N. J., Reynolds, E. C., *J. Chromatogr. B* 1997, *699*, 133–147.

[11] Šolínová, V., Kašička, V., Koval, D., Hlaváček, J., *Electrophoresis* 2004, *25*, 2299–2308.

[12] Jalali-Heravi, M., Shen, Y., Hassanisadi, M., Khaledi, M. G., *J. Chromatogr. A* 2005, *1096*, 58–68.

[13] Xin, Y., Mitchell, H., Cameron, H., Allison, S. A., *J. Phys. Chem. B* 2006, *110*, 1038–1045.

[14] Pei, H., Xin, Y., Allison, S. A., *J. Sep. Sci.* 2008, *31*, 555–564.

[15] Piaggio, M. V., Peirotti, M. B., Deiber, J. A., *Electrophoresis* 2005, *26*, 3232–3246.

[16] Piaggio, M. V., Peirotti, M. B., Deiber, J. A., *Electrophoresis* 2006, *27*, 4631–4647.

[17] Peirotti, M. B., Piaggio, M. V., Deiber, J. A., *J. Sep. Sci.* 2008, *31*, 548–554.

[18] Linderstrøm-Lang, K. C., *Trav. Lab Carlsberg* 1924, *15*, 1–29.

[19] Menon, M. K., Zydney, A. L., *Anal. Chem.* 2000, *72*, 5714–5717.

[20] Gitlin, I., Mayer, M., Whitesides, G. M., *J. Phys. Chem. B* 2003, *107*, 1466–1472.

[21] Allison, S. A., Carbeck, J. D., Chen, C., Burkes, F., *J. Phys. Chem. B* 2004, *108*, 4516–4524.

[22] Piaggio, M. V., Peirotti, M. B., Deiber, J. A., *Electrophoresis* 2007, *28*, 3658–3673.

[23] Sharma, U., Negin, R. S., Carbeck, J. D., *J. Phys. Chem. B* 2003, *107*, 4653–4666.

[24] Sharma, U., Carbeck, J. D., *Electrophoresis* 2005, *26*, 2086–2091.

[25] Beroza, P., Fredkin, D. R., Okamura, M. Y., Feher, G., *Proc. Natl. Acad. Sci. USA* 1991, *88*, 5804–5808.

[26] Mosher, R. A., Gebauer, P., Thormann, W., *J. Chromatogr.* 1993, *638*, 155–164.

[27] You, T. J., Bashford, D., *Biophys. J.* 1995, *69*, 1721–1733.

[28] Antosiewicz, J., McCammnon, J. A., Gilson, M. K., *Biochemistry* 1996, *35*, 7819–7833.

[29] Beroza, P., Fredkin, D. R., *J. Comp. Chem.* 1996, *17*, 1229–1243.

[30] Schaefer, M., Sommer, M., Karplus, M., *J. Phys. Chem. B* 1997, *101*, 1663–1683.

[31] Piaggio, M. V., Peirotti, M. B., Deiber, J. A., *Electrophoresis* 2007, *28*, 2223–2234.

[32] Tanford, Ch., *Physical Chemistry of Macromolecules*, Wiley, USA 1961, pp. 317–390.

[33] Creighton, T. E., *Proteins: Structures and Molecular Properties*, W. H. Freeman and Company, New York 1993, pp. 265–269.

[34] Bashford, D., *Front. Biosci.* 2004, *9*, 1082–1099.

[35] Russell, W. B., Saville, D. A., Schowalter, W. R., *Colloidal Dispersions*, Cambridge University Press, Cambridge, UK 1989, pp. 109–111 and 215–231.

[36] Piaggio, M. V., Deiber, J. A., *Lat. Am. Appl. Res.* 2003, *33*, 261–268.

[37] Happel, J., Brenner, H., *Low Reynolds number hydrodynamics*, Printice-Hall, Englewod Cliffs, NJ, 1965, pp. 205–207.

[38] Kim, S., Karrila, S. J. *Microhydrodynamics: Principles and Selected Applications*, Buttterworth-Heinemann, USA 1991, pp. 61–69.

[39] Fair, M. C., Anderson, J. L., *J. Colloid Interface Sci.* 1989, *127*, 388–400.

[40] Probstein, R. F. *Physicochemical Hydrodynamics*, Butterworths, New York 1989, pp. 107–114.

[41] Yoon, B. J., Kim, S., *J. Colloid Interface Sci.* 1989, *128*, 275–288.

[42] Kuntz, I. D., *J. Am. Chem. Soc.* 1971, *93*, 514–516.

[43] Creighton, T. E., (Ed.), *Protein Folding*, W. H. Freeman and Company, New York 1992, pp. 1–350.

[44] Harding, S. E., *Biophys. Chem.* 2001, *93*, 87–91.

[45] Levy, Y., Onuchic, J. N., *Proc Natl Acad Sci* 2004, *101*, 3325–3326.

[46] Kálmán, F., Ma, S., Fox, R. O., Horváth, C., *J. Chromatogr. A* 1995, *705*, 135–154.

[47] Sharma, U., Carbeck, J. D., in: Strege, M. A., Lagu, A. L. (Eds.), *Capillary Electrophoresis of Proteins and Peptides. Methods in Molecular Biology*, Humana Press, Totowa 2004, pp. 189–216.

[48] Zhou, H. X., *Biophys. Chem.* 2001, *93*, 171–179.

[49] Durchschlag, H., Zipper, P., *Biophys. Chem.* 2001, *93*, 141–157.

[50] García de la Torre, J., Huertas, M. L., Carrasco, B., *Biophys. J.* 2000, *78*, 719–730.

[51] García de la Torre, J., *Biophys. Chem.* 2001, *93*, 159–170.

[52] Lebowitz, J., Lewis, M. S., Schuck, P., *Protein Sci.* 2002, *11*, 2067–2079.

[53] Atkins, P. W., *Physical Chemistry*, Oxford University Press, Oxford 1995, pp. 757–759.

[54] O'Brien, R. W., White, L. R., *J. Chem. Soc. Faraday Trans.* 1978, *74*, 1607–1626.

[55] Allison, S. A., *Macromolecules* 1996, *29*, 7391–7401.

[56] Allison, S. A., *Biophys. Chem.* 2001, *93*, 197–213.

[57] Kim, J. Y., Ahn, S. H., Kang, S. T., Yoon, B. J., *J. Colloid Interface Sci.* 2006, *299*, 486–492.

[58] Brenner H., *Chem. Eng. Sci.* 1964, *19*, 519–539.

