Comparative Study of Analytical Techniques for Determining Protein Charge

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ABSTRACT: As interest in high-concentration protein formulations has increased, it has become apparent that routine, accurate protein charge measurements are necessary. There are several techniques for charge measurement, and a comparison of the methods is needed. The electrophoretic mobility, effective charge, and Debye–Hückel–Henry charge have been determined for bovine serum albumin, and human serum albumin. Three different electrophoretic methods were used to measure the electrophoretic mobility: capillary electrophoresis, electrophoretic light scattering, and membrane confined electrophoresis. In addition, the effective charge was measured directly using steady-state electrophoresis. Measurements made at different NaCl concentrations, pH, and temperatures allow comparison with previous charge estimates based on electrophoresis, Donnan equilibrium, and pH titration. Similar charge estimates are obtained by all of the methods. The strengths and limitations of each technique are discussed, as are some general considerations about protein charge and charge determination. © 2015 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 104:2123–2131, 2015
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INTRODUCTION

Many therapeutic proteins, especially at high concentration, may be prone to phase separation (aggregates, gels, and emulsions) and high viscosity. These unfavorable solution behaviors, which pose challenges for manufacturing, drug safety, and drug delivery, are a reflection of the colloidal properties of proteins.[1] Protein colloidal properties are controlled by proximity energies, which are principally electrostatic in origin. Charge–charge repulsion is the only long-range proximity energy that maintains protein solubility and that can overcome the attractive forces that lead to high viscosities. Therefore, it is important to have routine ways to measure protein charge.

Presented here is a comparison of measurements of the electrophoretic mobility, μ, net effective charge, z_{eff}, and the Debye–Hückel–Henry charge, z_{DHH}, for bovine serum albumin (BSA) and human serum albumin (HSA) made under identical solvent conditions using three different types of electrophoretic instruments, capillary electrophoresis (CE), electrophoretic light scattering (ELS), and membrane-confined electrophoresis (MCE). These results are compared with proton titration data,[2] Donnan equilibrium measurements,[3] and previous CE measurements,[4] as well as theoretical charge estimates computed from amino acid composition and from X-ray structure. The results are discussed with respect to how the charge estimates compare made using the different methods. The assumptions made for each method and strengths and weaknesses of each electrophoretic method are also discussed.

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BACKGROUND

Because there is renewed interest in making protein charge measurements, and the fact that many protein scientists may not have much experience with charge or with charge determination by electrophoresis, some background information may be useful.

Macromolecular Charge

Molecular charge is a fundamental property that directly influences protein structure, stability, solubility, and interactions with other macromolecules.[5–7] Rooted in the primary structure, the charge on a protein is a system property as it is affected by the solvent composition, pH, dielectric constant, and temperature. The actual protein charge may differ substantially from the charge calculated by summing up the charge on each of its ionizable groups, as these calculations only account for H^+ binding and it is known that proteins may bind other ions, particularly anions.[2,8–10]

Two types of ion binding are recognized, site bound and territorially bound. Site-bound ions are coordinated with the protein structure by specific bonds. These ions are fixed spatially and may be visible in an X-ray or nuclear magnetic resonance (NMR) structure. What is not depicted in an X-ray or NMR structure, but is important to protein net charge, are the territorially bound ions. These ions are not bound to a specific site. Instead, they are confined to regions of high-charge density on the protein surface in a manner similar to the "condensed ions" on a polyelectrolyte. The relevant parameter for what constitutes a high-charge density is the Bjerrum length, which is the distance separating two like-sign charges resulting in a potential energy in their vicinity comparable to the thermal energy, $k_{\mathrm{B}}T$, where k_{B} is the Boltzmann constant and T is the absolute temperature. In physiological solutions, Bjerrum length is 2–3 Å. Conversely, if two charges are closer together than

the Bjerrum length, then a counterion will be associated with them; and if there is a patch of charge on the protein surface, sufficient counterions will bind until the average charge separation equals or exceeds the Bjerrum length. Because territorial ion localization requires a high-charge density, the extent and location of territorially bound ions is affected by a protein's structure.[10–12] Territorially bound ions are characterized by their relative insensitivity to the bulk solvent ion concentration and by their close proximity to the protein surface (i.e., they are confined within the solvation layer).[10] Even though territorially bound ions may exchange freely with solvent ions, they do not dissociate from the macromolecule. Because they must move with the macroion, thereby not contributing significantly to its osmotic potential, they are an integral part of the protein's "charge structure."

Site-bound and territorially bound ions are distinct from those ions that make up the "counterion cloud" (i.e., the Debye–Hückel cloud) that forms as the solvent response to the protein's net charge. Instead, the Debye–Hückel cloud describes an imbalance of counterion and coion concentrations in vicinity of the protein. Importantly, the charge density distribution of the ion cloud depends on the solvent ion concentration and extends out from the protein surface several Angstroms into the surrounding solvent.[5,10] In short, the counterion cloud can be considered a solvent response to the presence of the charged protein, and the counterions that make up Debye–Hückel cloud are a consequence of the protein net charge rather than being an integral part of the protein charge.

Often the terms "charge" and "valence" are used interchangeably. The fundamental charge on a proton, Q_p, is 1.602×10^{-19} C (coulomb, SI units). The valence of a protein, z, is the unitless ratio of its charge, Q, divided by the fundamental charge, that is, $z = Q/Q_p$. So, a protein with a valence of +5 has a net charge of $+8.1 \times 10^{-19}$ C. Both the charge and the valence are signed quantities, so that a protein having a valence of −5 would carry a charge of -8.1×10^{-19} C.

The experimental quantities useful for charge determination are the electrophoretic mobility, μ in $cm^2/V\text{-}s$, (the ratio of the velocity of a molecule, in cm/s, to the electric field, in V/cm) and the effective valence, $z_{eff} = \frac{\mu}{fQ_p}$, where f is the translational friction coefficient, determined experimentally from either sedimentation or diffusion measurement and Q_p is the proton fundamental charge.[13] Both μ and z_{eff} include the effects of the Debye–Hückel ion shielding and of the "electrophoretic effect," which results from the distortion of the electric field in the vicinity of the nonconducting particle and from the transport of the ion atmosphere in the vicinity of the protein.[7,14] Both ion shielding and the electrophoretic effect reduce the electrophoretic mobility. Consequently, μ and z_{eff} do not distinguish between bound ions and the Debye–Hückel cloud. Two values calculated from μ and z_{eff} adjust for these effects, the zeta potential, ζ, and the Debye–Hückel–Henry valence z_{DHH}. In visualizing the difference between these two descriptions of a protein's charge properties, it is useful to consider the ζ potential as the electrical potential difference between the shear surface surrounding a spherical particle and the bulk solvent, where the potential difference is spread uniformly over the nonconducting particle surface, and z_{DHH} (actually $z_{DHH}{}^*Q_p$) as the charge at the center of the spherical, nonconducting particle that gives rise to the potential difference. These two quantities adjust μ for different effects. The zeta potential, ζ(in millivolts), corrects for the effects of the field distortion and counterion flow through Henry's function, H (below). Values of ζ potential are used widely to describe the charge on larger particles (e.g., pigments, latexes, etc.), and its derivation stems from the study of the electrophoretic behavior of macroscopic particles. The relationship between ζ and $\mu = \mu \frac{3\eta}{2DH}$, takes into account the solution viscosity (η) and the solvent dielectric constant, D. However, the calculation of ζ does not take into account the effects of Debye–Hückel shielding. On the contrary, Z_{DHH} also adjusts for the solvent shielding through the Debye–Hückel approximation, $Z_{DHH} = \frac{(1+k_D a)}{H} = \mu f \frac{(1+k_D a)}{HQ_p}$, where k_D is the inverse Debye length (in cm^{-1}), which depends on the temperature and the square root of the ionic strength, and a is the sum of the Stokes radii of the protein and its counterion.[13] The major difference between ζ and Z_{DHH} is that Z_{DHH} adjusts z_{eff} for the effects of the Debye–Hückel cloud, whereas ζ does not. In other words, z_{DHH} describes the protein charge, including any bound ions, while excluding the effects of the solvent ion cloud. In general, z_{DHH} is a more intuitive and useful description of the protein charge than ζ and will be used in this paper.

Henry's function, which is used to correct the mobility for the electrophoretic effects, depends on the unitless product $k_D a$ (i.e., $H = H(k_D a)$, and ranges from 1.0 to 1.5 as $k_D a$ ranges from 10^{-4} to 10^4. Our calculations use the approximation proposed by Moody et al.,[15] which is accurate to within 1% over the full range. Most proteins (including BSA, HSA, and IgGs) have hydrodynamic radii less than 13 nm, so that values of $k_D a$ are less than 5 in solvents having ionic strengths greater than 1 mM. For physiological solvents, a good first approximation is that $1+k_D a$ is approximately 3 and H is approximately 1.06, so Z_{DHH} is roughly threefold larger than Z_{eff}. Although Z_{DHH} is only an approximation, its accuracy seems to be within the uncertainty of the experimental measurements of charge and model calculations.[13]

Charge Determination by Electrophoresis

The processes involved in electrophoresis are made complex by the coupled flow of the ions in the electric field, and the effect the coupling has on the electric field.[14,16,17] However, there are four general considerations to keep in mind: (1) although a neutral protein will not move in an electric field, only neutral objects can move in the electric field. Although this may seem paradoxical, the electric field does not operate on individual particles. Rather, it operates on fluid volumes whose size is not fixed by a particle's van der Waals edges. In fact, the edges of the volume are bounded a diffuse layer that, at physiological salt concentrations, extends a few Angstroms out from the particle's van der Waals edge. The resulting volume has an overall net charge of zero. Net charge separation over distances greater than a few Angstroms is energetically very unfavorable, and adjustments back to a neutral condition will occur on a submicrosecond time scale.[18] As a consequence, at the fields used in electrophoresis experiments, it is more accurate to consider electrophoretic motion a biased diffusion process.

(2) Over short time scales, the distance moved by diffusion exceeds that moved by electrophoresis. Again, this may seem counterintuitive. However, the distance moved by electrophoresis is linearly proportional with time (i.e., doubling the time of electrophoresis doubles the distance moved) and the distance moved by diffusion is proportional to the square root of time (Fig. 1), so that at times less than a few seconds, a 5-nm protein having $\mu = 1 \times 10^{-4} \frac{cm^2}{V_s}$ diffuses further than it moves by