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Measuring zeta potential of protein nano-particles using electroacoustics

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ABSTRACT

Electroacoustic spectroscopy offers a simple way for measuring the zeta potential of proteins in physiological solutions with high ionic strength. Ultrasound as a driving force does not generate the heat effects which complicate traditional electrophoretic measurements at high ionic strength. In addition, measurements can be conducted with concentrated protein dispersions without dilution, as is required by electrophoretic methods. This paper presents results for electroacoustic measurements of 5 wt.% bovine serum albumin suspended in aqueous solutions. In these suspensions the proteins are not completely dissolved; they form nano-particles with a median size of about 180 nm. We studied the dependence of zeta potential on ionic strength within a wide range of salt molarities, up to as high as 0.5 mol/L. Dialysis was used for performing measurements at lower ionic strength range. We also conducted pH titrations of this system and titrations with Ca^{2+} ions. Our results agree well with published data for samples where such data is available.

1. Introduction

There is a very detailed review describing the gradual progress made in the studying of the electric properties of proteins, valency in particular, published 10 years ago by Winzor [1]. It covers almost a century's worth of research, beginning with pioneering work by Tiselius' group in the 1930s [2–4] and Abramson in the 1940s [5,6]. Despite all of these efforts, Winzor called the valency of proteins still an "...elusive parameter..." At the same time, he stressed the importance, and even urgency, in resolving this problem as "...a prerequisite for complete understanding of physiological control at the molecular level ..." [1].

He discussed the following methods:

1. computation of charge from the sequence data and set of p*K* values;
2. estimation of protein charge by pH titration;
3. estimation of protein charge from the Donnan distribution of charges;
4. electrophoretic methods:
   a. moving boundary electrophoresis
   b. paper electrophoresis
   c. gel electrophoresis
   d. capillary zone electrophoresis
   e. equilibrium electrophoresis

After detailed analyses of these methods, Winzor had critical comments about all of them. First of all, he objected to the first two methods on the grounds that protein charge depends not only upon the pH, but also on buffer/electrolyte composition. In addition, dissociation of any ionizable group depends on the overall charge and its distribution, which is practically impossible to take into account within computational models. Finally, he concluded that "...there is no realistic alternative to experimental measurement of protein valency...," which leaves us with methods 3 and 4.

From these two methods, Winzor prefers the one based on the Donnan effect [7]: number 3. He argues that this method is the least dependent on modeling the geometrical shape of the proteins. Electrophoretic methods, in contrast, require a protein geometric model for calculating protein charge from the measured electrophoretic mobility.

Unfortunately, the Donnan effect method has its own flaws. First of all, it requires substantial amounts of protein, which are often not available. Secondly, membrane potentials generated during dialysis experiments are very small, which would affect the precision of the protein charge characterization.

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Perhaps these are the reasons why electrophoretic methods have been more in use during last decade, since Winzor's review was published. However, instead of the electrophoretic methods mentioned by Winzor [1], a new one has become the most popular: electrophoretic light scattering (number 4*f* following the numbering started above). This method is described in detail in ISO Standard 13099, Part 2 [8]. There are many papers that present data using this method, for instance papers [9,10] being just some examples.

Unfortunately, this method suffers some drawbacks as well. Firstly, electrophoretic measurements of physiological solutions at high ionic strength are complicated by heat effects. High ionic strength leads to high electric current, which generates substantial heat. This effect could affect the state of the protein molecules. In addition, it creates heat convection that masks electrophoretic motion.

The second problem is associated with the calculation of protein valency from measured electrophoretic mobility. We discuss this aspect of electrophoretic light scattering here because the electroacoustic method that we suggest allows for resolution of this problem, to some degree.

Interpretation of electrophoretic measurements should be conducted according to ISO Standard 13099, Part 1 [11], which was approved by leading experts in the field from many countries. According to this document, the most widely used theory for the electrophoretic mobility, created by Smoluchowski [12], yields the following simple relationship between electrophoretic mobility μ and electrokinetic zeta potential ζ:

$$\mu = \frac{\varepsilon_m \varepsilon_0 \zeta}{\eta} \tag{1}$$

where ε_0 and ε_m are the dielectric permittivities of a vacuum and the liquid, respectively, and η is the dynamic viscosity of the liquid.

This theory has one marvelous feature: electrophoretic mobility is independent of particle size, shape, and volume fraction, *if* this theory is valid. This is the result of the geometrical similarity between hydrodynamic and electric fields within the framework of Smoluchowski's theory [13,14]. This often goes unrecognized. Nevertheless, even without recognition of this important fact, Smoluchowski's theory is built into the software of all modern commercial instruments for zeta potential measurements.

Unfortunately, this remarkable theory has a limited range of applicability. This was one of the interpretation obstacles mentioned by Winzor in his review. According to ISO Standard 13099, Part 1, the range of applicability for Smoluchowski's theory is restricted by the following 4 conditions:

The first requirement is that the electric double layer (DL) surrounding the protein molecules (DL) must be much thinner than the characteristic size of the protein. This can be expressed as the inequality:

$$\kappa a \gg 1 \tag{2}$$

where κ is reciprocal of Debye length, and a is protein radius.

The second requirement is a negligible contribution of the surface conductivity, which is expressed as a small dimensionless Dukhin number *Du*:

$$Du \ll 1 \tag{3}$$

The third requirement is that the particle–liquid interface does not conduct normal electric current between phases. This condition is valid for non-conducting particles.

Finally, the fourth condition limits volume fraction of particles φ. It requires that the double layers of separate particles do not overlap, which leads to the following restriction on the volume fraction [11,16]:

$$\varphi < \varphi_{over} = \frac{0.52}{[1 + (1/\kappa a)]^3} \tag{4}$$

Commercial instruments with software based on Smoluchowski's theory almost always ignore these restrictions. As a result, many papers in which these instruments were utilized present ζ-potential values for systems where Smoluchowski's theory is not valid. This is especially important for proteins due to their small size.

Condition 2 of the thin double layer scenario imposes the most restriction on the applicability of Smoluchowski's theory for proteins. Parameter κa increases with increasing ionic strength. Therefore, the most favorable conditions for Smoluchowski's theory with regard to protein characterization would be in physiological saline with an ionic strength of 0.154 mol/L. Debye length is about 1 nm in such a solution. However, even at these most favorable conditions, Smoluchowski's theory is not valid for many proteins due to their small size. The geometric radius of proteins can be estimated as Stokes radius; it is usually on the scale of a few nanometers. For instance, the radius of *bovine serum albumin* (BSA) is 3.48 nm [15]. Therefore, parameter κa is only about 3 for BSA in saline solution. It becomes smaller and smaller at lower ionic strength. Smoluchowski's theory is not valid for such low κa values, according to ISO 13099-1, which presents other theories that must be used instead. Unfortunately, these other theories lose geometry independence, which is the characteristic of Smoluchowski's theory that made it desirable for studying proteins.

The situation is actually even more complicated, because ζ-potential calculation from electrophoretic mobility is only the first step in determining valency. The second step would be to employ theory for the double layer. ISO 13099-1 refers to DL theories that are valid for small κa values, but they are only valid for spherical particles with well defined homogeneous surfaces. It is not clear what range of error might be introduced by these models.

We offer here a method that allows for resolution of both problems mentioned above (heat effects and interpretation complexity), at least in some systems.

This novel method is based on electroacoustics, which is mentioned in ISO Standard 13099-1 as an alternative to electrokinetics for determining ζ-potential. Electroacoustic methods are suitable for characterizing concentrated dispersions without requiring dilution. There are two versions of electroacoustics, depending on the nature of the driving force applied for moving particles relative to the liquid. The version that relies on ultrasound for this purpose seems more attractive for proteins because it resolves the problem with sample heating if the liquid has high ionic strength.

Such method is called colloid vibration current (CVI), according to ISO 13099-1. A detailed description of the theory, verification experiments, and many applications can be found in the book [16]. We use here a commercially available instrument, which is described below. This instrument is capable of measuring CVI in concentrated dispersions. The software then calculates the electrophoretic mobility μ from CVI using following equation:

$$\mathrm{CVI} = \mu \frac{\varphi(\rho_p - \rho_s)}{\rho_s} \nabla P \tag{5}$$

where ρ_s and ρ_p are densities of the dispersion and of the particles, respectively, and P is a pressure in the driving ultrasound wave.

We would like to stress here that electrophoretic mobility can be measured at very high ionic strengths because the sample heating phenomenon that complicates electrophoretic light scattering is completely eliminated. Particles are driven by ultrasound, not an electric field. Propagation of ultrasound through the system is practically indifferent to the ionic strength. This method was applied for