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CRITICAL INSIGHT

Strengths and Weaknesses of Molecular Simulations of Electrosprayed Droplets

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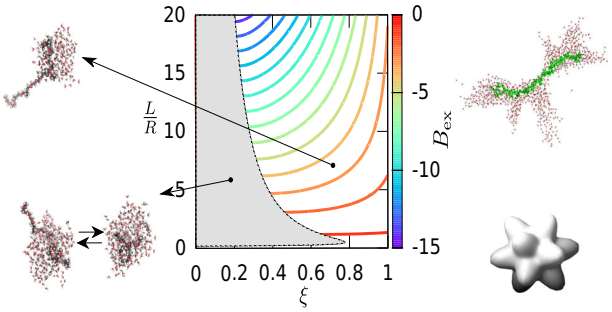

Abstract. The origin and the magnitude of the charge in a macroion are critical questions in mass spectrometry analysis coupled to electrospray and other ionization techniques that transfer analytes from the bulk solution into the gaseous phase via droplets. In many circumstances, it is the later stages of the existence of a macroion in the containing solvent drop before the detection that determines the final charge state. Experimental characterization of small (with linear dimensions of several nanometers) and short-lived droplets is quite challenging. Molecular simulations in principle may provide insight exactly in this challenging for experiments regime. We discuss the strengths and weaknesses of the molecular modeling of electrosprayed droplets using molecular dynamics. We illustrate the limitations of the molecular modeling in the analysis of large macroions and specifically proteins away from their native states.

Keywords: Droplet-macroion interactions, Molecular modeling, Solvation, Charge-induced instability, Rayleigh instability, Charged proteins, macroion extrusion mechanisms

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Introduction

Charged liquid droplets that carry macroions from the bulk solution into the gaseous phase for mass spectrometry (MS) analysis appear as intermediate states in many ionization techniques [8, 39, 49, 63, 79], among which are electrospray (ESI) [23, 83], sonic spray (SS) [35], thermospray [7], and possibly the matrix-assisted ionization (MAI) technique [22, 58]. Advances in MS coupled to droplet generating ionization techniques have extended the usage of MS beyond the traditional analytical chemistry field into the analysis of highly complex systems such as protein assemblies [32, 44, 47] and colloids [1]. In the last half decade, development of ion mobility mass spectrometry coupled to ESI has emerged as a promising technique for detecting details in conformational changes of proteins during melting in the gaseous state and conformations originating from the bulk solution [25, 26, 60, 73, 74]. A key question in all the ionization techniques coupled to MS is how the finally detected macroion acquires its charge [27, 40, 45, 81].

Significant progress in understanding the droplet chemistry has been achieved in the ESI-MS field since it is one of the earliest and broadly used ionization methods [23, 83]. J. Fenn and co-workers studied the charging mechanism of the macromolecules in droplets [28, 29, 51, 83] by interpreting the outcomes of ESI-MS experiments. Their findings, even though very insightful, lacked direct evidence. Molecular modeling, on the other hand, is well suited to study fast molecular reactions such as evaporation events and conformational changes of macroions in systems with several tens of thousands molecules often inaccessible to experimental techniques. Thus, molecular modeling can provide insight into molecular details that were missing in older investigations. In this article, we will focus on the strengths and weaknesses of the computational methods for finding the relation between the solvation and charging of macroions in droplets.

Since the charged droplets evolve through a cascade of fission events, the first question to address is their stability.

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The study of the stability of a conducting spherical droplet is found in the seminal works of Lord Rayleigh [19, 34, 62]. Under the assumption that a conducting droplet of charge Ze (where e is the elementary charge) and radius R undergoes small shape fluctuations relative to a sphere, the condition for stability is

$$Z^2e^2 < 64\pi^2\sigma\varepsilon_0R^3 \quad (1)$$

where ε_0 is the permittivity of vacuo and σ the droplet surface tension. When equality holds in Eq. (1) ($Z^2e^2 = 64\pi^2\sigma\varepsilon_0R^3$), the droplet is at the Rayleigh limit. Hereafter, we will denote the charge of the droplet at the Rayleigh limit as Z_re. Above the Rayleigh ($Z^2e^2 > 64\pi^2\sigma\varepsilon_0R^3$), a spherical droplet cannot exist. Thus, a conducting droplet will fragment below the Rayleigh limit. A conducting droplet that is composed of free ions and a non-fissile macroion may become dielectric when all the free charge has been released. A dielectric droplet has its own "Rayleigh limit" [55] different from that of the conducting droplet. At the "Rayleigh limit" [55] of the dielectric droplet, provided that the macroion does not release charge, the spherical shape of the droplet becomes unstable. Then the unstable spherical shape transforms into a new shape so as the system is found in an energy minimum [13, 19, 41, 55]. The new stable shape appears by the formation of conical solvent protrusions along the body of the macroion as evidenced by atomistic [13, 72] and continuum modeling [55].

To set the stage, we should recognize that the nanodrop chemistry is more complicated than that of the bulk solution. The differences between the two systems have been described in previous work [21]. Here, we will focus on one major difference, which is the constant change of the size of the droplet as the macroion travels from the source to the detector. A number of droplet characteristics vary during the process such as droplet composition, macroion charge state, temperature, and droplet pressure. The transfer of the macroion from the source to the mass spectrometer is inherently a non-equilibrium process dealing with a dynamic system at every stage.

We think that it is helpful to distinguish three stages in droplet evolution [45]. An initial drop size in which solvent composition as well as the state of a macroion is very close to that of the bulk solution. It is expected that the chemistry in these system sizes will follow thermodynamic theories. An intermediate stage where the droplet contains a number of ions that is comparable to the charge of the macroion. We think that this intermediate stage is the most interesting because at this stage, the droplet environment has some very unique properties. One can demonstrate theoretically that in this stage, the macroion charge is the highest [46]. At this stage, the macroion charge is determined to a large degree by the chemical equilibrium between the droplet buffer and the macroion. The final stage, detected by an instrument, is the result of evaporation of the remaining solvent and escape of the remaining free ions. Modeling of the chemical equilibrium equations demonstrates that along with the escape of the free ions, the macroion must release charges as well. However, we think that this final stage is very short-lived; hence, one may not use chemical mass action equations to determine the macroion charge. In this latest stage, it may be possible that the final charge is determined by the interplay between kinetics of the evaporation and protonation reactions [46, 67, 69].

Prior research, based on experimental observations, has led to a number of important findings. De la Mora provided a particularly insightful version of the charged residue model (CRM) that went beyond the model of Dole [23] by inferring the final charge state of a globular protein [33, 40, 50, 65, 82]. The prediction was based on the fact that the final charge state of a globular protein is determined by the charge that a corresponding spherical droplet enveloping the macroion at the Rayleigh limit can hold. This prediction assumes that globular proteins retain their conformations until the last stage of the droplet existence. The CRM model assumptions are to be compared with the alternate view that suggests that proteins may reduce their charge during their drying in evaporating droplets [46, 67, 69] as was discussed earlier. Fenn and de la Mora [28, 29, 50] have suggested that certain linear macroions, such as poly (ethylene glycol) (PEG) and unstructured proteins, may extrude from a droplet when charged. It has been suggested that the extrusion of a linear macroion can possibly explain the high charge states of linear macroions [36, 37]. The question that we discuss in the following sections is to what extent the molecular modeling can be used to relate the macroion solvation in a droplet to the final charge state of the macroion.

Challenges in MD Simulations of Charged Droplets

Molecular dynamics (MD) simulations provide a glimpse into the realistic evolution of atomistic systems. In principle, MD simulations can mimic the outcome of a mass spectrometry experiment if one takes into account all the interactions of a droplet with the environment as it moves from a source to a detector. We can monitor in great detail the droplet evolution as if one observes the atomistic motions of the experiment through a magnifying glass. However, in practice, there are serious limitations in its usage both of computational and theoretical nature. We distinguish two types of simulations that one can use (i) study of a droplet at equilibrium that provides the average properties of a droplet state for a particular droplet size [48, 54] and (ii) non-equilibrium simulations that attempt to capture the entirety of the evaporation fission process [14, 78]. Non-equilibrium MD runs without a thermostat to maintain the temperature of a droplet are evaporative cooling runs [43]. The non-equilibrium simulations may lead to kinetic trapping of the macroion conformations.The equilibrium simulations are based on the assumption that there is a time-scale separation between molecular equilibration and macroion dynamics. These types of simulations allow dramatic acceleration of the simulation rate by eliminating the simulations of slow evaporation events. We assume that in between the evaporation events, the droplet is at equilibrium. Technically, the

simulations are performed by placing the droplet in a cavity or a simulation box with periodic boundary conditions. The equilibrium simulations may mimic the canonical or the microcanonical ensemble. The advantages of equilibrium simulations are that the outcome does not depend on the initial conditions and the results are universal in the sense that they are reproducible and robust. In equilibrium simulations, the conditions of temperature and vapor pressure can be controlled. Kinetic trapping may be avoided in equilibrium simulations by employing advanced sampling techniques beyond direct MD runs [3]. In the equilibrium runs, the rates of conformational changes of macromolecules or of other processes can be determined and compared with the droplet evaporation rate [56]. This comparison infers whether the processes found in the equilibrium simulations may occur during droplet evaporation.

Non-equilibrium simulations are performed by placing the droplet in vacuo and observing the evolution of the droplet containing the macroion [14]. Non-equilibrium runs without a thermostat are evaporative cooling runs. Intuitively, the outcome of such an experiment provides information on a pathway of an actual electrospray experiment. The results of each trajectory may depend on the initial conditions. As a consequence, accurate non-equilibrium simulations of charged droplets require detailed description of the droplet environment: pressure and temperature of the buffer gas, velocity of the droplet in the buffer, the solvent vapor pressure, and other parameters of the mass spectrometer. These parameters are frequently unavailable and differ between experiments. Computational details of the two approaches can be found in refs. [21, 56].

Initially, we will describe some of the challenges that are common in both types of simulations.

Treatment of Electrostatic Interactions

The first challenge is that molecular simulations of a macroion in a charged droplet are somewhat challenging relative to that of the bulk solution simulations for a number of reasons. Elongated droplet shapes, conical protrusions on the droplet surface, and instabilities (fission of droplets) result from the long-range Coulombic forces that need to be calculated either explicitly or indirectly through a use of a multipole expansion method. The direct calculations of the Coulomb potential have an N^2 scaling with the number (N) of partial charges making it impractical for large systems. The multipole expansion methods, on the other hand, require sophisticated space binning techniques as the droplet occupies only a small fraction of the available space in a simulation box. The inefficiency of molecular simulations prevents us to explore microscopic systems especially when the processes we study are related to shape fluctuations and instabilities. It is likely that when we look into a local behavior in the interior of the droplet, where shape fluctuations may not play a role, truncation of electrostatic interactions is possible. However, the results of the simulations with and without cutoffs have to be compared in test systems before any efficient method of dealing with the electrostatic interactions is selected. Because of these limitations in atomistic simulations, we can only explore currently nanoscopic droplets with diameters on the order of 10 nm [72] composed of a few tens of thousands of solvent molecules.

Limitations on the Simulation Time and System Size

The second challenge that arises from the first is that in a molecular simulation, we only explore a very short path in droplet's lifetime relative to the experimental droplet sizes and timescales. For instance, molecular simulations of a protein require a careful and well thought-out preparation of the initial ensemble. The steps comprise determination of the protonation state of the protein that corresponds to the acidity conditions of the particular droplet size [46], protonation of the titratable residues, and finding the corresponding conformation for the particular protonation state [56]. An additional complication that naturally arises is that different charge distributions on the protein may correspond to the same protonation state [56]. The determination of the different protonation states and their effect in the protein conformation requires its own study. The history of the droplet evolution up to the size accessible to numerical experiments may also be important. This is especially true in non-equilibrium simulations because the natural initial state of simulations has to be determined from non-equilibrium dynamic events. The initial conditions of non-equilibrium simulations lack this information [46].

Sampling of Macroion Conformations

The third challenge is the sampling of protein conformations. In the study, we should take into account the fact that the droplet environment has different composition and pH from those of the parent bulk solution [46]. Therefore, the protonation state of a protein in the very small droplets appearing in the latest stage of the evaporation process may change [46] depending on the dynamics of the various processes (proton transfer reactions [15], solvent [54], and ion evaporation [12]). One of the challenges is that the force fields used in simulations of proteins have not been optimized to capture the conformational changes for the various charge states, besides the compact native state under physiological conditions. One of the practical difficulties of the simulations is that a realistic initial conformation of the protein corresponding to this size of a droplet and pH is not known [46]. There have been attempts to develop custom force field to study protein conformational changes in acidic environment [4] but the adoption of the force field has been rather limited. If one uses artificially prepared conformations, the results are inconclusive. For instance, in our simulations, we tested aqueous droplets with an embedded myoglobin, atomistically modeled, in charge state + 17 since it has been used as an example by several experimental groups that propose a protein extrusion mechanism [2, 36, 37, 42]. The details of the simulations are presented in the Supplementary Material Sec. S1. We found that a + 17 charged myoglobin in bulk solution maintains its native structure in simulations performed for few tens of nanoseconds. In a droplet environment, this compact (native) conformation is maintained up to the

latest stage of the droplet lifetime, where denaturing leads to rapid extension of the protein. If we start the simulations from an extended myoglobin observed in the gaseous state, which is an artificially prepared conformation for testing purposes, we observed that the extended conformation does not change throughout the simulation.

Droplet Physical State

The fourth challenge is the relation between the experimental temperature [30, 76] and the simulated droplet temperature. The origins of the problems are the following: (i) the nanodrops are finite-sized systems; therefore, a bulk phase diagram of the solvent will not even be valid to begin with [10, 80] and (ii) the force fields of the protein conformations have been optimized for the native state under physiological conditions; therefore, to observe any conformational changes of proteins elevated temperature is required [57]. This elevated temperature is the result of the inadequacy of the force field; thus, the temperature that induces the conformation change may not be related to the temperature that an actual conformational change may occur. A possible way to obtain an estimate of the temperature to be used in the simulations is by matching the evaporation rate of the model solvent to that of the experimental evaporation rate. It has been reported that nanosprayed droplets evaporate within micro-seconds [81]. Simulations of droplets composed of 2000 TIP3P H_2O molecules evaporate in a rate approximately 1 H_2O molecule/3 ps at temperature 370 K [54]. Even though there are considerable efforts to determine the droplet temperature [30, 43, 76], a more exact knowledge of the experimental evaporation rate may help to set the temperature in the simulations.

Modeling of the Charging Mechanisms

In the simulations of electrosprayed droplets, we should consider that the droplet environment has different composition from that of the parent bulk solution. Unlike the constant native environment of a macromolecule in bulk solution, the droplet environment is dynamic and fast changing. Depending on the dynamics of the various processes (proton transfer reactions [15], solvent [54], and ion evaporation [12]), the emerged macromolecule may possess different charge from that in the parent solution.

Herein lies a major difficulty in predicting the charged state of a macroion. An accurate prediction of the charge state should be based on a reliable model of the droplet environment for droplets with radii in 10–100 nm range. There are various models of the droplet behavior on the last stages of their life. The competing models are heating due to viscous drug forces [59] and evaporative cooling [24, 43].

Non-dissociative force fields cannot capture the protonation mechanisms. Therefore, for practical simulations, alternate methods have been devised. Cationization instead of protonation of proteins might be a possible alternative under certain circumstances. If the ions are not intended to react but only to provide additional charge, to study for instance charge-induced instabilities then using either Na^+ ions or protons in the form of hydronium ions may lead to the same droplet fluctuations provided that the droplet surface tension is not affected substantially by the ions. This is supported by the Rayleigh limit expression, which does not depend on the nature of the ions (besides the surface tension effect). On the other hand, the rate of release of Na^+ vs that of hydronium ions may be different. Consequently, the protonation state of a macroion may change. When the Na^+ ions are used to replace protons in their acid-base reactions, several apparent differences arise. On the one hand, a proton reacts with a basic site of a protein by forming a covalent bond that is described by quantum mechanics. Of course, the covalent bond may dissociate and one may have proton transfer reactions [84]. On the other hand, an alkali ion interacts with a basic site by electrostatic interactions using classical mechanics. The consequence of this difference is that a Na^+ is not exclusively associated with an ionizable group of the side-chains. There is a distribution of Na^+ ions surrounding the macroion where some of them are further away from the ionizable groups. The non-bound Na^+ ions may be released from the droplet at different rates from the covalently bound protons. This may affect the final charge state of the macroion. A discussion is presented in the Supplementary Material Sec. S2 for the difference between protonation and cationization of a protein. Na^+ ions also will form ion pairs with other ions and with themselves. Simulations have indicated that salt adducts may change the overall conformation of the macroion in the gaseous state as it has been shown for dsDNA [71]. Another major difficulty lies in the calculations of the equilibrium constants of the participating species. While pK_a values for protonation reactions are relatively well studied, one cannot say the same for the cationization reactions. The difference between cationization and protonation requires a detailed study and a careful consideration if the results of cationization are to be related to those of protonation.

On the fly, we can observe directly and with confidence to the force fields the charging by sodiation (lithiation) of PEG [11, 14, 53]. PEG does not undergo protonation reactions; therefore, the observation of the cationization mechanism can be close to reality. The results of the maximum charge state of PEGs of several lengths are in agreement with the data of Fenn [83]. The rich phenomenology of the PEG extrusion mechanisms and the dependence on temperature and length of the PEG have been presented in refs. [11, 14, 53]. We emphasize that extrusion of the charged PEG does not imply complete detachment from the droplet. Temperature affects the degree of extrusion of a linear macroion as well as its charge state [14, 70]. Moreover, several extrusion mechanisms have been identified in ref. [20]. Nucleic acids also undergo protonation reactions; however, their overall conformational changes are not substantial upon protonation [5, 71]. Therefore, if we examine the stability of dsDNA due to its charge, we may replace protonation with sodiation. We note that the PEG and DNA in an aqueous droplet represent two extreme cases of macroions in terms of their flexibility. Protein degree of flexibility lies in between that of PEG and DNA. Proteins may

change their conformation upon protonation. As discussed previously, this change is still challenging to be captured by MD simulations.

Promising approaches to study protonation of macroions are quantum mechanics/molecular mechanics (QM/MM) [66], the reactive force field (ReaxFF) [64], and multi-scale modeling [56]. QM/MM can be used to study local changes of proton transfer between a few water molecules and a protein site. QM/MM is suitable for implementation in equilibrium droplet simulations in a cavity. Estimates of protonation rates [15] at equilibrium can be compared with the droplet evaporation rates, and thus infer whether the reaction may occur during the droplet evolution. The ReaxFF is expected to be applicable in equilibrium simulations in a cavity and in the constantly and globally changing environment of a droplet mimicked in a non-equilibrium simulation. Since these methods require more computationally expensive modeling than molecular mechanics, the size of droplets that can be studied will be even smaller than that studied by molecular mechanics force fields (discussed earlier).

Multi-scale modeling that involves macroscopic equations to determine the protein charge state and atomistic MD simulations of the protonated protein is another approach. The macroscopic approach is based on the simulations of the complex chemical equilibria in charged droplets. A number of software packages have been developed [31, 38, 61] based on stochastic simulations of chemical reactions. The multi-scale modeling allows one to take advantage of the better sampling provided by equilibrium simulations and the implementation of advanced sampling techniques [3]. We may infer whether the process under investigation takes place in the evaporating droplet by comparison with the droplet evaporation rate. Currently, the multi-scale modeling seems to be a more efficient approach relative to QM/MM and ReaxFF modeling in terms of providing natural initial states for simulations, more efficient sampling of macroion conformations, and ability to study larger systems.

Strengths of MD Simulations

There are several ways that molecular simulations can be used to extract robust information about macroiondroplet interactions. Equilibrium simulations of droplets may be used to find the local structure of the solvent around a macroion and ion pairs. The dynamics of formation of contact and solvent separation ionic species can be determined even though it is quite complicated for more than two species [16]. A separate calculation of the evaporation rate can be used for comparison of the rates. Simulations can also detect formation of adducts via equilibrium or non-equilibrium simulations [71].

Robust data can also be obtained for the equilibrium solvation patterns of macromolecules. These motifs are presented in Fig. 1. In Fig. 1a, we depict the extrusion of a macroion from a droplet [13, 14]. We found that this scenario is followed by a realistically modeled sodiated (or lithiated) poly (ethylene glycol) (PEG) molecule [11, 14, 51, 53, 77, 83]. Figure 1b shows that a droplet may separate into sub-droplets where each of which includes a part of the macroion. Each sub-droplet is found below the Rayleigh limit ($X < 1$). We have named this droplet shape "pearl-necklace" [18] conformation. Figure 1b shows the example of two sub-droplets at the termini of the heat shock protein 12 (Hsp12) from *Saccharomyces cerevisiae* (RCSB PDB [6] code 2LJL [75]) in the charge state + 15 *e* at 350 K, that we have simulated. Figure 1c shows conical protrusions of a droplet containing a macroion with bound charge (i.e., a non-fissile macroion). The state of a droplet with conical protrusions is stable [13, 55] and arises when the system passes from a charge-induced instability cross-point. The "pearl-necklace" conformation and the extrusion of a linear macroion from a droplet are not the results of charge-induced instabilities. For a spherical central ion, the conical protrusions take a regular "star" shape as shown in Fig. 1d [53]. We are confident about the existence of these solvation patterns because they have been consistently found for different macroions and solvents. These solvation patterns have been identified in equilibrium [55] and non-equilibrium simulations [71]. The question associated with the solvation patterns is how they are related to the charge state of the macroion. The general solvation motifs develop because of the fundamental physical interactions; therefore, it is expected that they will participate in the mechanism of droplet disintegration, and finally in the manner in which the charge of the macroion is determined.

Relation of Droplet Shapes to Charging Mechanisms

Since protonation mechanisms cannot be observed directly so far, one may infer the charging mechanism of macroions by a combination of analytical theory and molecular simulations. It has been found experimentally that certain proteins have higher charge than the Rayleigh limit of the corresponding spherical aqueous droplet composed of the protein and a few layers of water. Examples of such proteins are the "supercharged" denatured proteins and others such as the PHBH in [9], 2Ubq (ubiquitin dimer) in [68], Urease $\alpha_{18}\beta_{18}$, Urease $\alpha_{24}\beta_{24}$ in [33], and aerolysin k in [81]. These proteins could not have been found in a spherical droplet above the Rayleigh limit because such droplets cannot exist. We believe that the supercharged proteins appear in intermediate sized droplets with the total charge of approximately double that of the charge of a macroion [46].

This division of charge half on the protein and half as free charge has been discussed in detail in ref. [46]. The charge state of ubiquitin (Ub) and the ubiquitin-associated (UbA) domain from DNA-damage-inducible 1 protein (Ddi1) (RCSB PDB [6] code 2MRO [52]) and the droplet pH in different buffer concentrations are shown in Fig. 2a, b. Figure 2a, b shows that the minimum pH corresponds to the maximum charge state of the protein, which is approximately half of the charge of the droplet. Analysis of the chemical equilibria in the presence of a buffer demonstrates that the macroion obtains the maximum charge in intermediate-sized droplets. In the course of subsequent evaporation, the droplet loses all the free ions through fission events. The charge of the protein may change by release of protons [46, 67, 69].

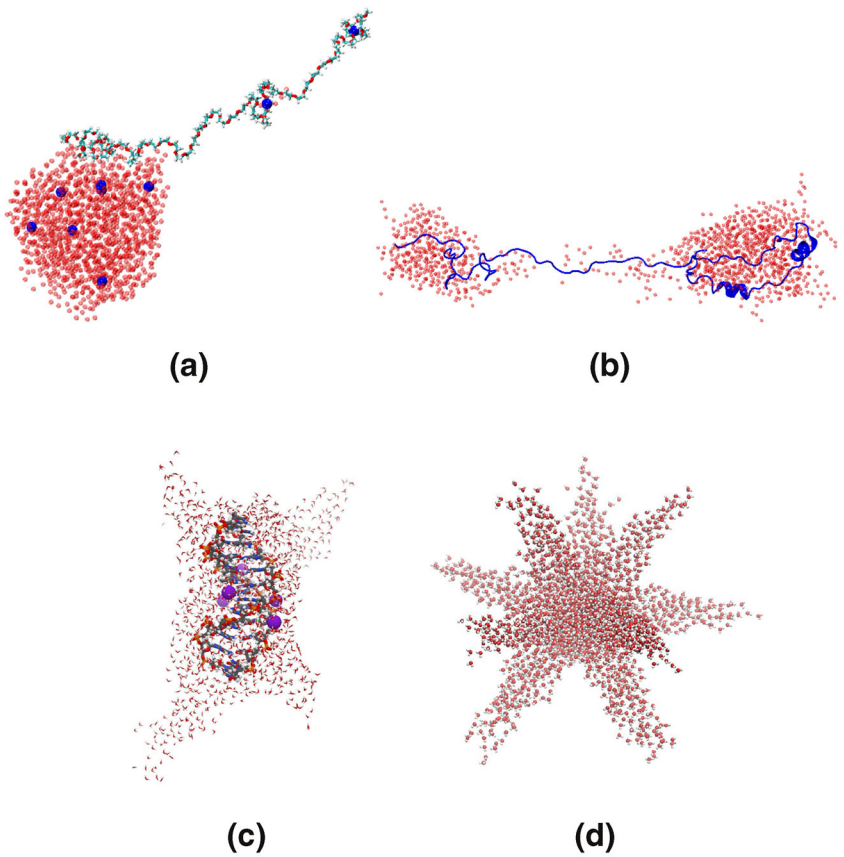

Figure 1. Classes of stable solvation states of a macroion in a droplet. These classes emerge at certain values of the droplet charge-squared-to-volume ratio. The classes are demonstrated by typical snapshots taken from simulations of atomistically modeled systems. (**a**) Gradual extrusion of a linear macroion from a droplet. It is demonstrated by the extrusion of a poly (ethylene glycol) from an aqueous droplet [14, 17]. The blue spheres represent Na^+ ions, and the red dots represent H_2O molecules. (**b**) "Pearl-necklace" droplet conformation. The "pearl-necklace" conformation is demonstrated by a droplet comprised approximately 1200 water molecules and heat shock protein 12 (Hsp12) from *Saccharomyces cerevisiae* (RCSB PDB [6] code 2LJL [75]) in the charge state +15. (**c**) Formation of conical protrusions of the solvent surrounding a macroion. A droplet comprised water (red dots), a negatively charged 20mer (ds stands for double stranded) dsDNA and Na^+ ions (purple spheres) [71]. (**d**) A ten-point "star"-shaped droplet with a central spherical macroion (the structure is three-dimensional). Details are described in the text

Once the free charges have been released and the protein has not been de-protonated, it is possible for the droplet to evolve to a "spiky" structure (Fig. 2c–f). If the formation of the spikes is faster than the protein deprotonation, then the release of protons from the protein in order to reduce its charge is prevented by the emerged ordered structure of the surrounding solvent. The ions can be released only via the "spike" pathways and not from the troughs. Considering that the solvent conical protrusion may not extend out from the charged amino acids and also they are variable in location, their presence may inhibit the proton release from the protein [56]. This mechanism may lead to supercharged proteins.

The presented analysis suggests that the final protein charge state may be determined by the release of protons from proteins that have been already found in a high charge state for the specific conditions of the droplet.

As it has been described earlier by de la Mora [50] and later by Hogan [36, 37] and other authors [45], one of the still unresolved questions is the high charge state of denatured proteins sprayed from an acidic solution. These proteins have been found to be "supercharged" [36, 37, 45]. It has been proposed [36, 37] that partial desolvation of protein segments may occur instead of charge carrier emission. The charge segments that desorb may carry the charge away; therefore, this charge does not participate into the charging of the remaining portion of the protein found in the droplet. As was discussed in the previous sections, extrusion of an unstructured protein from a droplet is rather difficult to be evidenced directly by simulations. Of course, there are the trivial cases of protein evaporation such as that of a poly (valine) that may contain a single protonated lysine along its chain at elevated temperature and under certain conditions. This is the case of a highly hydrophobic protein that lies on the surface of a droplet and has a small charge. Analytical modeling of the extrusion mechanism of a linear charged macroion for any solvent-macroion interactions has been derived. The model is discussed in refs. [17, 20] and it is summarized in the Supplementary Material Sec. S3. The predictive power of the model is found in the

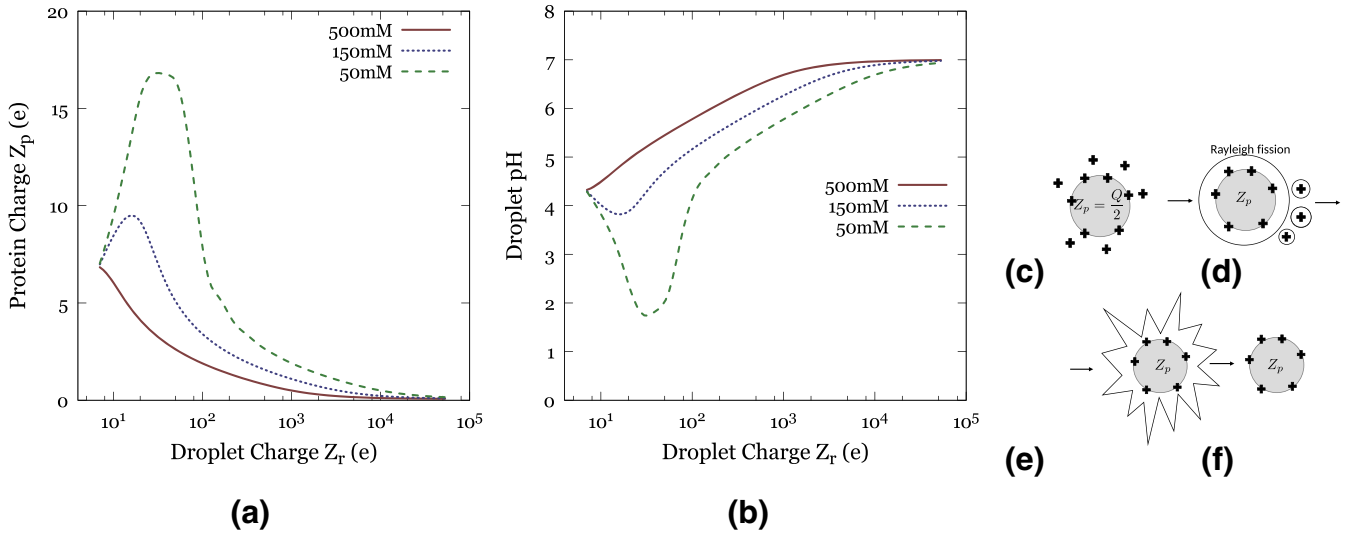

Figure 2. (**a**) The charge state of 2MRO [52] (details in the text) Zp (e) vs. droplet charge Zr (e) for various concentrations of CH3COONH4. (**b**) Droplet pH vs. droplet charge Zr (e) for the same concentrations of CH3COONH4 as those in (**a**). (**c**–**f**) Schematic representation of the charging of a protein that has obtained its maximum charge state in the intermediate sized droplet. (**c**) The gray region represents the protein that carries approximately half of the droplet charge. (**d**) The single charges are released by Rayleigh fission. (**e**) If the rate of deprotonation is slower than the rate of spike formation, the droplet develops a "spiky" structure. (**f**) The droplet dries out

universal parameter that introduces, which is solvation energy over the charge density squared of the macroion. The model predicts that macroions with the same value of the universal parameter follow the same extrusion path. The analytical model does not exclude the possibility of the extrusion of proteins; however, we have not directly detected extrusion of proteins by atomistic modeling thus far.

In the Supplementary Material Sec. S1, we present several examples of solvation of proteins of different degree of hydrophilicity to be compared with the solvation of a sodiated PEG. It is found that the ionizable groups of several typical proteins that we examined remain quite solvated. This indicates that it is difficult to observe protein extrusion similar to that of PEG within the feasible simulation times. Moreover, in the proteins we examined, the release of the free ions occurs faster than the release of the charged protein. In addition, we remark that protein conformational changes that may accompany the extrusion process are quite slow to be detected within a feasible simulation time.

Conclusion

Atomistic modeling of electrosprayed droplets using molecular dynamics still poses significant challenges. We discussed the limitations of molecular modeling in terms of atomistic force fields and challenges in the sampling of a macroion conformation, preparation of a protein model, choice of a protonation state, statistical representation of the outcomes, and the droplet physical state. Despite the challenges, molecular simulations and analytical theory have provided several robust results thus far. The analytical theory of the extension of the Rayleigh theory for dielectric droplets [55] predicts the onset of instability as a function of the droplet dielectric constant. This theory is applicable to droplets that contain a charged protein or nucleic acids and no free ions. In addition to the CRM for macroion charging, we advocate the theory that the charge of a macroion is determined by the solvation properties of the macroion or, more accurately, by the buffer-macroion chemical equilibrium in the intermediate sized droplets. In the latest stage of a droplet's lifetime, the final charge state of the macroion may be determined by deprotonation rates. The rates of the reactions will be determined by the specific details of the analyte molecules and the structure of the solvent. If the deprotonation rates of the macroion are slow, then the droplets may form conical protrusions and supercharging of the macroions may take place.

The extrusion mechanisms of atomistically modeled poly (ethylene glycol) [11, 14, 17, 77] has provided a direct example of how the charging mechanism of a macroion is coupled to a linear macroion extrusion mechanism. The same way of coupling may not hold for proteins. The coupling of the charging to the release of unstructured proteins from a droplet needs to be explored further. P. Kebarle in one of his well-known reviews [40] writes "the IEM is experimentally well-supported for small (in) organic ions. However, the theoretical derivation of the model does not apply for very large ions such as proteins. For these macromolecular species, the CRM is much more plausible ...". Since the 2009 review, there has been progress. An analytical theory for the extrusion of linear macroions, which is the analogue of IEM for simple ions, has been developed [17]. The theory has predictive power because it has provided the universal parameter for the extrusion mechanism of macroions (Supplementary Material Sec. S3), insight into various extrusion mechanisms that result from the interplay of solvation and electrostatic energy and also the conditions for macroion extrusion in terms of length of the macroion, charge, and size of the droplet [17, 20]. Since the 2009 review, additional mechanisms

to CRM for protein charging have been proposed where protein deprotonation in the latest droplet sizes may take place [46, 67, 69]. A still open question is whether atomistically modeled proteins extrude from aqueous droplets. Because of the difficulties in modeling discussed in this article, this is still a rather challenging question to answer with confidence.

Simulating the protonation reactions in a droplet environment is another obstacle. Modeling using multi-scale methods [21, 54, 56], quantum mechanics/molecular mechanics (QM/MM) [66] and reactive force field (ReaxFF) [64] are promising approaches to this problem. The multi-scale approach seems the most promising approach at the moment for molecular mechanics simulations.

We believe that the combined efforts of experiments and computations have come close to resolve the fundamental mechanisms of charging of macroions. A classification of the mechanisms has already been done to a certain extent [18, 20], and it is anticipated to be completed in the near future.

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