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A Repulsive Field: Advances in the electrostatics of the ion atmosphere

Vincent B. Chu,

Department of Applied Physics, Stanford University, GLAM, McCullough 318, 476 Lomita Mall, Stanford, CA 94305

Yu Bai,

136 Fleming Bldg., Department of Chemistry, University of Houston, Houston, TX 77004

Jan Lipfert,

Kavli Institute of Nanoscience, Faculty of Applied Sciences, Delft University of Technology, Lorentzweg 1, 2628 CJ Delft, The Netherlands

Daniel Herschlag, and

Department of Biochemistry, Stanford University, B400, Beckman Center, Stanford, CA 94305

Sebastian Doniach

Department of Physics and Applied Physics, Stanford University, GLAM, McCullough 308, 476 Lomita Mall, Stanford, CA 94305

Vincent B. Chu: vincentc@stanford.edu; Yu Bai: yubai@kitten.chem.uh.edu; Jan Lipfert: J.Lipfert@tudelft.nl; Daniel Herschlag: herschla@stanford.edu; Sebastian Doniach: doniach@drizzle.stanford.edu

Summary

The large electrostatic repulsion arising from the negatively-charged backbone of RNA molecules presents a large barrier to folding. Solution counterions assist in the folding process by screening this electrostatic repulsion. While early research interpreted the effect of these counterions in terms of an empirical ligand-binding model, theories based on physical models have supplanted them and revised our view of the roles that ions play in folding. Instead of specific ion-binding sites, most ions in solution interact inside an “ion atmosphere” – a fluctuating cloud of non-specifically associated ions surrounding any charged molecule. Recent advances in experiments have begun the task of characterizing the ion atmosphere, yielding valuable data that has revealed deficiencies in Poisson-Boltzmann theory, the most widely-used theory of the ion atmosphere. The continued development of experiments will help guide the development of improved theories, with the ultimate goal of understanding RNA folding and function and nucleic acid/protein interactions from a quantitative perspective.

RNA molecules fulfill an astounding array of roles in the cell. Beyond their “traditional” roles as messengers between transcription and translation, RNA molecules play essential functional roles in the cell, catalyzing highly specific reactions and regulating gene expression through

Correspondence to: Daniel Herschlag, herschla@stanford.edu; Sebastian Doniach, doniach@drizzle.stanford.edu.

Conflicts of Interest

The authors report no conflict of interest.

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novel mechanisms and pathways [1,2]. Acquisition of a specific, three-dimensional structure through folding is an initial and obligate step for many of these RNAs. The electrostatic contribution to RNA folding is enormous and cannot be discounted. The negative charge carried by each nucleotide residue contributes a repulsive force which folding must overcome; for instance, folding a ~400 nucleotide RNA in the absence of counterions requires overcoming an astounding electrostatic barrier of approximately 10^3 kcal/mol [3]!

Historically, early research was focused on the seemingly “mysterious” roles played by solution counterions such as Mg^{2+} in RNA folding. Experimentally, RNA folding was found to depend crucially on salt concentration. This dependence led many researchers to view RNA folding through a ligand-binding model; folding progress was described using the empirical Hill model in terms of specific binding sites for Mg^{2+} and other positively charged cations. The parameters obtained from fitting the Hill equation to folding progress curves were interpreted as the number of specific binding sites and the cooperativity of the folding transition (e.g., [4–6]).

However, our understanding of the roles played by solution ions in RNA folding and other nucleic acid systems has been revolutionized by the application of models derived from physical theory. Using Poisson-Boltzmann (PB) theory, a simplified model of the interactions between RNA and solution counterions, as a starting point, Draper and coworkers clearly articulated that the majority of ions interact with RNA not through specific ion-binding sites, but through an “ion atmosphere” – a diffuse cloud of non-specifically associated ions that surrounds any charged molecule in solution [7].

This updated view divides RNA-ion interactions across a continuum with two extremes: ions loosely associated with RNA within a mobile and fluctuating ion atmosphere, and a small number of specific ion-binding sites where ions from solution are localized by regions of strongly negative electrostatic potential or specific contacts. Ions in the atmosphere remain hydrated and are in fast exchange with the bulk solvent [8]. They serve to stabilize RNA structure by screening the electrostatic repulsion between RNA domains. On the other end of the spectrum, specifically-bound ions are often at least partially dehydrated and exchange more slowly. In fortunate cases they can be observed crystallographically. Functionally, site-bound ions may stabilize local regions of high charge density, bring together structural elements, and participate in catalysis [7,9–11].

In this review, we focus on recent breakthroughs in our experimental and theoretical understanding of the ion atmosphere. In particular, we discuss experimental probes that now allow characterization of the ion atmosphere and its energetic effects on RNA folding and nucleic acid systems. We also discuss assessments of electrostatic theories of the ion atmosphere and current challenges in the quantitative modeling of the electrostatic effects in nucleic acid systems.

The need for a physical model of the ion atmosphere

The sigmoidal dependence of many folding transitions on ionic concentration is well-fit by the Hill equation, a model of cooperative ligand binding to a molecule that is familiar to many biochemists. However, its use in describing RNA folding is completely empirical. The parameters obtained from fitting the Hill equation to folding curves should be viewed as purely descriptive and their usual significance as the number of binding sites and cooperativity is lost when applied to RNA folding [7]. In fact, simple physical arguments show that the Hill model cannot possibly be a correct model of ion-RNA interactions.

Physics demands that the charge-weighted number of ions associated with an RNA molecule balance its net charge –i.e., the overall charge on the system must be zero. However, fitting

the Hill equation to a folding transition reveals far fewer binding sites than is required to satisfy charge neutrality. Further, such a model would imply that charge neutrality is not satisfied at low ionic concentration. Clearly, the Hill model cannot be a complete model of ion association for the majority of ions involved in an RNA system. The fundamental requirement for charge neutrality has also been directly verified by recent experiments (see “Characterizing the ion atmosphere”).

The use of the empirical Hill model as a description of folding limits our understanding of the ion atmosphere and its energetic effects. Deep understanding requires an approach whose foundation is based upon theories that accurately reflect physical reality. Development of such models has the potential to guide our intuition into the basic properties of the ion atmosphere and make quantitative predictions. As a foundational model of the ion atmosphere, Draper and coworkers have employed PB theory; using this theory they found that ion association to nucleic acids –previously interpreted using the Hill model–could be reinterpreted as ions associating inside an ion atmosphere [12–14].

In the past ten years, driven by its computational simplicity and the release of popular software packages to solve it, PB theory has become the predominant theory of the ion atmosphere [12,15–17]. In the area of nucleic acids alone, PB has found wide application (e.g., [7,18–21]).

Nevertheless, like any model, PB contains simplifications and assumptions that render it inaccurate under certain conditions. In particular, PB theory assumes that the solvent behaves as a simple continuum dielectric, that solution ions are point-like in nature (i.e., occupy no volume), and that ions in solution do not explicitly interact with one another (often referred to as the “mean-field” approximation) [22,23]. Because the coulombic interaction between ions scales as the product of their valences, the effect of these ion-ion interactions is more pronounced in solutions containing multivalent ions. Physically, ion-ion interactions cause the ions in solution adopt “correlated” positions to reduce their mutual repulsion; these spatial correlations increase the ion density within the ion atmosphere and enhance their ability to screen electrostatic repulsion. For trivalent or tetravalent ions, correlation effects are responsible for ion-induced DNA condensation and overcharging phenomena in trivalent and tetravalent systems, effects which PB theory cannot possibly predict [22,24,25]. As described below, increasingly sophisticated comparisons between PB predictions and experiment are beginning to quantify the importance of these deficiencies and will hopefully spur the development of theories that go beyond PB to correct its defects.

Characterizing the ion atmosphere

The ephemeral nature of the ion atmosphere resists experimental probing by standard structural techniques of crystallography or NMR. Nevertheless, experimental measurements of the ion atmosphere are required, not only to establish its basic structure and characteristics, but also to critically assess various electrostatic theories that describe it. In spite of its fluctuating nature, direct measurements of the shape of the ion atmosphere are possible using anomalous small angle X-ray scattering (ASAXS). The difference in scattering at wavelengths off and on the resonant scattering “edge” of a particular ion species allows the scattering contribution from that species to be isolated. Thus, the scattering of a variety of monovalent and divalent ions surrounding nucleic acids can be directly measured [26,27]. As expected from basic electrostatic considerations, ion atmospheres composed of monovalent cations had twice as many ions as an atmosphere made up of divalent cations. Also as expected, the concentration of divalent cations decreases faster than monovalents as one moves away from the nucleic acid, consistent with the greater screening ability of cations of higher valence.

Finally, the results were roughly consistent with expectations from PB but suggested a need for a correction for ion size (see below).

The composition of the ion atmosphere has been probed using Buffer Exchange-Atomic Emission Spectroscopy (BE-AES). This technique essentially allows investigators to count the number of each ionic species present in a mixed-species ion atmosphere. In this technique, a sample of RNA is repeatedly equilibrated against a buffer solution at a given ionic concentration. Quantification of the number of ions in equivalent volumes of RNA-containing sample and buffer solution gives the number of excess ions attracted by the negative charge of the RNA. Quantification by AES provides great precision for such measurements, in part because multiple ion species in admixture can be assayed.

The number of excess ions associated in the ion atmosphere reveals the relative affinity of each ion species for the nucleic acid at equilibrium [28]. Similar data can also be obtained with varying degrees of sensitivity, convenience, completeness, and accuracy from atomic absorption spectroscopy, dye-indicator methods, and NMR line broadening [29,30,31 Grilley, 2006 #115].

BE-AES has been used to characterize the ion atmosphere surrounding model DNA duplexes. Such experiments have verified, with unprecedented accuracy, that the ion atmosphere completely neutralizes the net charge of a nucleic acid (Q_{duplex}) by attracting solution cations and repelling anions; thus, the local environment of a nucleic acid is enriched with positively charged counterions and depleted of anions (Eq. 1). The precise combination of cation enrichment and anion depletion used to achieve overall charge neutrality is dependent on the enthalpy and entropy of this process and is revealed by ion counting experiments.

$$Q_{\text{duplex}} + 2 N_{\text{Mg}} + N_{\text{Na}} - N_{\text{Cl}} = 0 \quad (1)$$

To gain intuition into this process, let us explore how the ion atmosphere neutralizes the charge of a simple 24 basepair DNA duplex (net charge $-46e$) with constant Mg^{2+} and varying Na^+ : “Low Na^+ ” (5 mM Mg^{2+} , 10 mM Na^+), “Medium Na^+ ” (5 mM Mg^{2+} , 100 mM Na^+), and “High Na^+ ” (5 mM Mg^{2+} , 1 M Na^+) (depicted in Figure 1A, B, and C). At the low Na^+ condition, charge neutrality is due to almost entirely to the excess Mg^{2+} . The great affinity of Mg^{2+} (relative to Na^+ and Cl^- depletion) can be explained using simple physical arguments. Localization of a single Mg^{2+} in the ion atmosphere neutralizes two negative duplex charges for the entropic cost of localizing a single ion; accomplishing the same neutralization with more Na^+ would require the higher entropic cost of localizing two ions in the ion atmosphere.

At the medium Na^+ condition, the increased Na^+ concentration has “competed” some of the Mg^{2+} away due to its 20-fold higher relative concentration in the bulk; under this condition, they contribute roughly equally to the charge neutralization. However, the loss of each Mg^{2+} from the atmosphere is not accompanied by a gain of exactly two additional Na^+ , due to the higher entropic cost of incorporating two additional Na^+ ions into the atmosphere. Instead, this entropic cost is reduced by incorporating fewer than 2 Na^+ into the ion atmosphere and shifting some of the neutralization to Cl^- exclusion.

At the high Na^+ condition, the relative concentration of Mg^{2+} in the bulk is so low that it does not participate significantly in charge neutralization. In this case, charge neutrality is satisfied through a combination of associated Na^+ and excluded Cl^- .

The highly quantitative data obtained from BE-AES provide powerful constraints that electrostatic theories must satisfy. Results from these studies on simple DNA duplexes indicate

that the size of the ion affects its affinity and consequently, its screening ability; such size effects are not considered in PB theory. Further, these studies indicate that PB theory significantly underestimates the number of Mg^{2+} associated in the ion atmosphere, indicating that Mg^{2+} is associated more strongly than PB theory predicts [28]. PB's failure to accurately account for divalent metal association has been attributed to its neglect of ion-ion interactions [24]. Neglecting these ion-ion interactions may lead to inaccuracies in the predicted energetic contribution of electrostatics in Mg^{2+} and other divalent metal ion solutions (see below).

Energetic tests of Poisson-Boltzmann theory

The screening conferred by the ion atmosphere strongly influences the ensemble of conformational states, dictating which conformations are likely to be present. The screening of the ion atmosphere (i.e., the ability to shield the repulsive forces arising from the negative charge of the RNA backbone) is determined by the length scale over which the negative charge of the RNA is neutralized by the ion atmosphere. At low ionic concentrations, the diffuseness of the ion atmosphere means that the charge is neutralized over long distances. Under these conditions, the negatively-charged domains of an RNA molecule interact strongly and repel, inhibiting them from approaching one another. At higher ionic concentrations, the charge is neutralized over short length scales; this enhanced screening allows the domains to come in close contact with substantially less cost in electrostatic energy, allowing formation of the hydrogen bonds that stabilize tertiary contacts [3,32–35].

While this view qualitatively explains how the ion atmosphere assists in the folding process, quantitative predictions from PB and other theories of the ion atmosphere must be compared against experimental data to assess how well they predict the energetic effects of the ion atmosphere on the conformational ensemble of RNAs. Accurate prediction of electrostatic energies will ultimately allow investigators to predict the distribution of conformers from energetic calculations at any given salt concentration.

The Draper lab has worked extensively on quantifying, both experimentally and theoretically, the energetic contribution of the ion atmosphere in folding in a variety of systems [11,13,36–41]. While they find reasonable agreement between PB predictions and experiment for a large range of ionic conditions, the complexity of the investigated systems and the assumptions inherent in the theoretical frameworks used may introduce uncertainties in the comparison between experiment and theory [42].

To address these uncertainties, Doniach, Herschlag and colleagues have taken a different approach. To study the electrostatic forces between helices and their modulation by the ion atmosphere, they used a tethered duplex system composed of two 12 basepair DNA duplexes joined by a neutral polyethylene glycol tether (Figure 2). Free of specific ion binding sites or complicated junction regions, the tethered duplex is designed to be a model system whose conformational ensemble is determined only by the electrostatic repulsion between its component duplexes and the conformational states of the neutral tether. The system's simplicity ensures that accurate modeling of its conformational ensemble is computationally tractable and transparent. The equilibrium conformational ensemble under a variety of ionic conditions was monitored by small angle X-ray scattering (SAXS) and directly compared to predictions derived from PB theory [3,42].

Studies on this system have strongly suggested that Mg^{2+} -induced attractive forces between helices do not play a substantial role in folding under standard *in vitro* folding conditions, a previously proposed driving force of conformational relaxation [3]. Furthermore, comparisons between PB predictions and SAXS experiments have shown that PB-derived energies are an adequate description of the screening provided by the ion atmosphere in monovalent salt solutions; however, PB significantly underestimates the screening efficiency of the ion

atmosphere for divalent ions (Figure 3). Again, these inaccuracies presumably stem from PB's neglect of ion-ion interactions, which are expected to help stabilize nucleic acid structure. Given the physiological importance of Mg^{2+} and other divalent ions, this inaccuracy represents a great deficiency in PB theory [42].

Future Directions

Much has been learned in recent years, but much work remains. The recent advances cited above suggest that the application of PB theory to physiologically important divalent systems must be reevaluated. Given the shortcomings of PB, theories that go beyond it to correct its deficiencies are clearly needed. Theorists are beginning to address this need, developing models that incorporate, to varying degrees, corrections that address PB's simplified treatment of the solvent, ion size, and ion-ion correlation [23,43–51]. Although many of these treatments are promising, progress in this field continues to be hampered by the lack of direct synthesis between theoretical predictions and experimental verification. The continuing dearth of simple, accessible software routines that allow predictions to be made for realistic molecular geometries will also continue to frustrate progress. Until these needs are addressed, PB theory – despite its shortcomings – will continue to be the *de facto* theory of the ion atmosphere. Whatever its flaws, PB analysis is still far superior to Hill analysis as it provides quantitatively correct predictions in monovalent solutions, and qualitatively correct predictions in divalent solutions.

Despite the value of simple model systems such as the tethered duplex for the assessment, development, and refinement of theoretical models, future challenges will undoubtedly be the application of these refined theories to realistic and biologically relevant systems. In this area much work has already been done to establish a basic framework that incorporates the effects of the ion atmosphere and specific ion binding in RNA folding [11,36,37]. However, this framework must be augmented with improved electrostatic theories and a proper treatment of how junction flexibility and tertiary contact formation contribute to folded RNA stability [42].

Although electrostatics is a necessary and indispensable component of our view of RNA folding, we must remind ourselves and our readers that it is but one of a number of fundamental physical forces that contribute to the stability of folded RNA, such as the hydrogen bonds that stabilize tertiary contacts, stacking interactions, the energetic effects of specifically-bound ions, and the conformational biases imposed by different junction regions in RNA. As electrostatic theories improve and our confidence in them grows, investigators will increasingly be called to apply these theories not only in more complicated systems, but in concert with models describing other fundamental forces. Ultimately, the integration of these forces will lead to a complete view of RNA folding, RNA function, and RNA-protein interactions.

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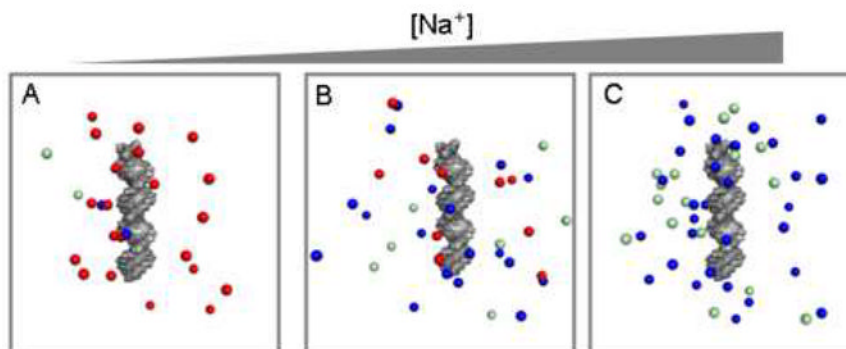
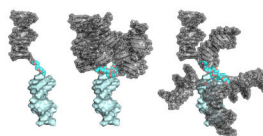


Figure 1.

This figure depicts “snapshots” of the ion atmosphere around a 24 base pair DNA duplex (net charge: $-46e$) as Na^+ is titrated into a fixed 5 mM Mg^{2+} background (with Cl^- co-ions). The sizes of the ions have been exaggerated for clarity. Charged objects in solution achieve charge neutrality by attracting cations (Mg^{2+} , red; Na^+ , blue) or excluding anions (Cl^- , pale green). A) At 10 mM Na^+ , the ion atmosphere is composed almost entirely of excess Mg^{2+} (21 excess Mg^{2+} , 2 excess Na^+ , and 2 excluded Cl^-). B) At 100 M Na^+ , the increasing availability of Na^+ in the bulk increases its presence in the ion atmosphere (10 Mg^{2+} , 19 Na^+ , 7 excluded Cl^-). C) At 1M Na^+ swamps out Mg^{2+} . Under these conditions, the charge neutralization is due essentially to 30 excess Na^+ and 16 excluded Cl^- . (Data adapted from reference [28])

**Figure 2.**

Left: The tethered duplex consists of two 12 base pair DNA duplexes joined by a polyethylene glycol tether and is an analog of the ubiquitous helix-junction-helix motifs found in all structured RNAs. Middle: Under low salt conditions, the large electrostatic repulsion forces the helices into mostly extended conformers. Right: At higher salt concentrations, the electrostatic penalty is screened, allowing a greater range of conformers, including conformers where the helices adopt a side-by-side arrangement.

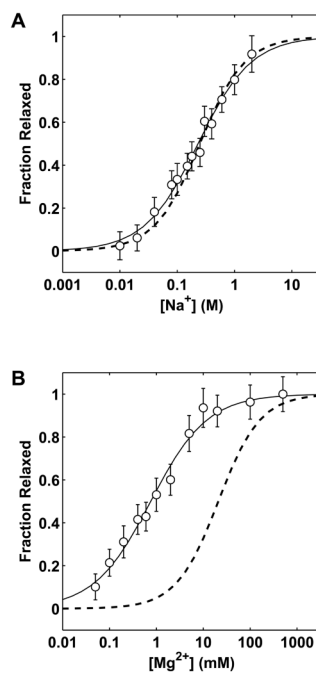


Figure 3. Structural relaxation of a simple tethered duplex in Na^+ (A) and Mg^{2+} (B). Fraction relaxed represents the degree of relaxation as measured by SAXS experiments (solid lines) and predicted by PB theory (dashed lines). Reproduced with permission from [42]. Copyright 2008 American Chemical Society.