Influence of salt and viral protein charge distribution on encapsidation of single-stranded viral RNA molecules

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We examine the limits on viral composition that are set by the electrostatic interactions affected by the charge on the viral proteins, the single-stranded viral RNA molecule and monovalent salt ions in the solution. Within the mean-field model of viral energetics we demonstrate the prime importance of the salt concentration for the assembly of a virus. We find that the encapsidation of the viral RNA molecule is thermodynamically suppressed in solutions with high concentrations of monovalent salt. This effect is significantly less important in viruses with proteins whose charge distribution protrudes into the interior of the capsid, leading to an increase in the stability of such viruses in solutions with high salt concentrations. The delocalization of positive charge on the capsid protein arms thus profoundly increases reliability of viral assembly in high-salt solutions.

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Viruses are a prime example of precise spontaneous assembly. It was more than fifty years ago since Fraenkel-Conrat and Williams demonstrated that fully infectious tobacco mosaic viruses (TMV) could be created simply by mixing the viral RNA molecules together with the viral proteins\textsuperscript{1}. Under the right conditions (pH and salinity), the viruses formed spontaneously, i.e. without any special external constraints.

There is no unanimous view concerning the physical interactions that guide the viral self-assembly. It is often supposed that specific interactions acting between the viral genome and its proteins guarantee the precise assembly. However, this cannot be the entire story since it has been demonstrated that (i) empty viral protein coatings (capsids) assemble, at least when the amount of salt in the bathing solution is large enough\textsuperscript{2,3}, and (ii) the filled virus-like particles form even when the viral genome is replaced by noncognate RNA molecules\textsuperscript{2,3}.

The role of salt in the viral assembly is of essential importance. Already in the early studies of TMV assembly\textsuperscript{1} it was found that the viral proteins can be assembled in capsid-like structures at sufficiently high salt concentrations even if the pH of the solution is high enough to prohibit the assembly in low ionic concentrations (or causes an alkaline degradation of the assembled viruses). It is experimentally documented\textsuperscript{2,3} that upon lowering the pH of the high-salt solution (> 0.8 M LiCl) with disassembled proteins and RNA of brome mosaic virus below pH 6.5, only empty viral capsids form. All these experimental findings clearly indicate that there is a nonspecific interaction of electrostatic origin acting between the viral proteins and RNA molecules. This interaction depends crucially on the concentration of salt ions in the bathing solution.

The aim of this letter is to decipher the role of salt in the assembly of viruses that contain single-stranded highly negatively charged (one elementary charge per nucleotide) RNA (ssRNA) molecule. The proteins of such viruses typically carry positive net charge\textsuperscript{6} at physiological pH. For many of these relatively simple viruses it has been experimentally demonstrated that they can be spontaneously assembled in vitro. Spontaneous assembly takes place only if the energy of the capsid-genome complex is favorable - as proposed already by Caspar and Klug\textsuperscript{6}. In the context of electrostatic interactions, this means that the number of charges on the ssRNA and the capsid must be related and this relation should depend also on the amount of added salt.

We approach the problem by representing the viral ssRNA as a generic flexible polyelectrolyte with effective monomer size $a$, and $pe$ charge per monomer, where $e$ is the electron charge and $0 < p < 1$. The polyelectrolyte concentration, $\Psi(r)^2$ and electrostatic potential, $\Phi(r)$, are treated as continuous real-valued fields that minimize the mean-field ground state dominance free energy of the polyelectrolyte/capsid/salt system\textsuperscript{2,3}, $F$, in the subspace of fixed total number of polyelectrolyte monomers, $N$, so that

$$F = \int f(r) d^3r - \mu \left( \int d^3r \Psi(r)^2 - N \right),$$

where $\mu$ is the Lagrange multiplier enforcing the condition of fixed number of monomers, and

$$f(r) = k_B T \left[ \frac{\alpha^2}{6} (\nabla \Psi(r))^2 + \frac{\nu}{2} \Psi(r)^4 \right] + \left[ ee^+ (r) - ee^- (r) - pe \Psi(r)^2 + \rho_p (r) \right] \Phi(r) - \frac{e \alpha}{2} (\nabla \Phi(r))^2$$

for the electrostatic contributions to the free energy.
Here $T$ is the temperature, $k_B$ is the Boltzmann constant, $c^\pm$ are the concentrations of $+$ and $-$ monovalent salt ions, with $c_0^i$ being their bulk concentrations, and $\mu^\pm$ their chemical potentials, $\epsilon_0$ is the permittivity of water, and $v$ is the (non-electrostatic) excluded volume of the polyelectrolyte chain. The density of charge located on the capsid proteins is denoted by $p_p(r)$. We shall consider this charge density to be fixed, i.e. we shall investigate the stability of the assembled capsids at fixed (physiological) pH. The variation of the free energy functional with respect to fields $\Psi$, $\Phi$ and $c^\pm$ yields two coupled non-linear equations - the generalized polyelectrolyte Poisson-Boltzmann equation\textsuperscript{10,11}, and the Edwards equation\textsuperscript{12}. These equations are solved numerically with the requirement that the polyelectrolyte density amplitude field vanishes at the interior capsid radius, $R$. It is known that the asphericity of ”spherical” (icosahedral) viruses increases with the mean radius of the virus\textsuperscript{12}. However, deviations from the perfectly spherical shape are generally small even for quite large viruses\textsuperscript{12}, thus our approximation of spherical symmetry is not expected to be a serious limitation. We do not explicitly account for the mechanical elasticity of the polyelectrolyte, i.e. our approach is tailored to flexible polyelectrolyte molecules, the ssRNA in particular. Our approach cannot account for the details of the RNA conformation, such as its branched and locally double stranded structure, or its possible dodecahedral ordering in the vicinity the capsid, a feature that has been investigated recently\textsuperscript{13,14,15}.

Since the size of empty capsids is usually the same as in the fully functional viruses (at least in a range of pH and salinity values\textsuperscript{12,13}), it is reasonable to fix the capsid radius at a prescribed value, corresponding to the preferred mean curvature of the empty capsid\textsuperscript{16,17} and to examine the energetics of the filled capsid depending on the amount and type of enclosed polyelectrolyte and the concentration of salt ions in the bathing solution. Figure\textsuperscript{1}a) displays the free energies of the polyelectrolyte/capsid/salt system as functions of the number of monomers ($N$) in the polyelectrolyte for three different salt concentrations in the bulk bathing solution. In this calculation, we have represented the protein charges as a uniformly charged infinitely thin spherical shell of surface charge density $\sigma = 0.4$ e/nm$^2$ and inner radius $R=12$ nm. This should be representative for typical ssRNA viruses\textsuperscript{16,17}. In Fig\textsuperscript{1}b) we represent the polyelectrolyte concentration profile for several polyelectrolyte lengths and for ”physiological” salt concentration of $c_0 = 100$ mM. There are several important messages that can be read directly of this figure. First of all, the energetics of viral capsids is profoundly influenced by the concentration of salt. Second, there is a critical number of monomers that can be thermodynamically packed within the capsid. This happens at the point when the total energy of the system becomes larger than the energy of the empty capsid ($N = 0$); in Fig.\textsuperscript{1} these are the points at which the full lines intersect with the dotted horizontal lines for given bulk concentrations of the salt. For polyelectrolytes larger than this critical size, formation of empty capsids is thermodynamically preferable. While it is generally easier to form capsids at elevated salt concentration (this is seen from the smaller values of the free energy at high salt, irrespective of the number of monomers), it is more difficult to form filled capsids. For low salt concentrations, the critical number of monomers is such that the total polyelectrolyte charge is about two times larger than the energy of the empty capsid ($c_0 = 10$ mM). For $c_0 = 700$ mM, it is only about 100, twelve times smaller than the critical number in low salt ($c_0 = 10$ mM). We

\begin{equation}
+ \sum_{i=\pm} \{k_B T \left[ c^i(r) \ln c^i(r) - c^i(r) - (c_0^i \ln c_0^i - c_0^i) \right] - \mu^i \left[ c^i(r) - c_0^i \right] \}.
\end{equation}
define the optimal number of monomers as the one that minimizes the free energy for a given salt concentration (in agreement with Ref. 16). In low-salt solutions, this happens when the total polyelectrolyte charge approximately equals the capsid charge, but in elevated salt, the optimal number of monomers decreases. In contrast to Ref. 16, we find that the optimal number of monomers is such that the magnitude of charge on the polyelectrolyte is always smaller than the protein charge.

These findings can be better understood by examining the polyelectrolyte density in the capsid [Fig. 1b)]. Due to attraction between the polyelectrolyte and the capsid, there is always a maximum in the polyelectrolyte concentration at a distance \( \xi \) from the capsid [distributions similar to that shown in Fig. 1b)] have been experimentally observed - see e.g. Ref. 7. When the number of monomers is larger than the optimal one, the polyelectrolyte density becomes finite throughout the capsid, filling the capsid core, although the maximum in the density close to the capsid is still distinguishable even for \( c_0 \) as large as 700 mM (not shown). At high salt, the electrostatic interactions are screened and the polyelectrolyte entropy becomes important in the total balance of free energy. In this regime, it becomes energetically favorable even for quite short (depending on exact amount of salt) polyelectrolyte to delocalize over the whole capsid as there is enough salt to efficiently screen the capsid charge even in the absence of the polyelectrolyte. We find that sub-optimal polyelectrolyte conformations are always such that the polyelectrolyte is located only within a shell close to the capsid, while the super-optimal conformations are extended throughout the whole capsid, irrespectively of the salt concentration. A similar result was found by authors of Ref. 13, who used a discretized version of a model akin to ours that does not include the effects of salt, however.

Not all ssRNA viruses can be represented by an infinitely thin shell of positive protein charge density. While this may be a reasonable approximation for viruses as dengue or yellow fever, it is certainly a poor approximation for e.g. cucumber mosaic virus (see Fig. 2), tomato aspermy virus and the much investigated cowpea chlorotic mottle virus. These viruses are known to have specifically shaped capsid proteins, so that their N-terminal tails are highly positively charged and stretched. When virus of this type is fully assembled, the capsid protein "arms" protrude into its interior. It has recently been suggested that the existence of highly basic capsid peptide arms can explain the proportionality between the net charge on the capsid proteins and the total length of the ssRNA viral genome. Here we are more interested in investigating whether such delocalization of the protein charge influences the dependence of stability of a virus on the salt concentration. We represent the capsid charge density as

\[
\rho_p(r) = \frac{Q_c}{4\pi r^2 \xi}, \quad R - \xi < r < R,
\]

and \( \rho_p(r) = 0 \) otherwise, i.e. we treat the capsid peptide arms as strongly stretched polyelectrolytes, but not necessarily of brush type, of length \( \xi \), carrying in total a charge \( Q_c \) per capsid. The actual charge distribution in real viruses depends on the amino acid content of the capsid peptide arms, but it is worth mentioning that results practically indistinguishable from those shown in Fig. 2 are obtained by assuming that \( \rho_p = \text{const.} \) for \( R - \xi < r < R \) and zero otherwise, so that the total protein charge is still \( Q_c \) (the robustness of the results is mostly due to the fact that \( \xi \ll R \)). Note that we do not account for steric repulsion acting between the capsid peptide arms and the viral ssRNA i.e. for the loss of interior capsid volume available to ssRNA resulting from the protrusion of pieces of capsid proteins into the capsid interior. Figure 3 is analogous to Fig. 1 but for capsid charge density represented by Eq. 3. The total charge on the capsid (\( Q_c = 724 e \)) is the same in these two cases studied. The arm length chosen (\( \xi = 2.5 \text{ nm} \)) should be representative of cucumber mosaic virus (see Fig. 2). Comparing Figs. 1 and 3 one can conclude that (i) when the polyelectrolyte length is optimal, the viruses with charge delocalized on the capsid arms are bound significantly stronger - this can be seen from smaller values of \( F \) achieved at the minimum when compared with the corresponding values in Fig. 1 (for \( c_0 = 100 \text{ mM} \), the difference in binding energies is about 450 \( k_B T \) per capsid), and (ii) the optimal length of the polyelectrolyte is significantly less influenced by the salt concentration (for \( c_0 = 10 \text{ mM}, 100 \text{ mM}, \text{ and} \ 700 \text{ mM}, \) the optimal monomer density...
numbers are 700, 650, and 550, respectively). Our second result is in rough agreement with the findings by Belyi and Muthukumar who estimate that the total ssRNA charge and the total capsid charge are equal up to a quantity of the order of 10 elementary charges for salt concentrations below about $c_0 = 100 \text{ mM}$. Our results thus bridge the two apparently contradictory previous attempts to describe the viral energetics and show that the spatial distribution of protein charge determines the important features of the energetics of viruses with regard to salt concentration. Note from Fig. 3b) that the thickness of the ssRNA "shell" is determined by the length of the protein arms, unlike in the case of infinitely thin shell of viral protein charge where it is determined by $a$ and $v$ parameters of the polyelectrolyte. For superoptimal polyelectrolyte lengths there appears a characteristic "two-humped" profile of the polyelectrolyte concentration which results from an interplay of the four length scales involved in this case – the $a$ and $v$ parameters of the polyelectrolyte, the length of the capsid arms, $\xi$ and the Debye-Hückel screening length that depends on the salt concentration.

In summary, our results show that the influence of salt on the energetics of viruses is quite important, especially for viruses whose positive charge on the capsid interior may be well represented as an infinitely thin shell. However, the delocalization of positive charge on the capsid protein arms profoundly increases the reliability of viral assembly in high-salt solutions. This effect also increases resistance of the assembled viruses towards disassembly in the solutions containing high concentration of (monovalent) salt. Our results strongly suggest that the highly charged delocalized capsid protein arms may offer an evolutionary advantage to viruses that have them. This does not conflict with the fact that the viruses we examined are very simple ones, since, as Caspar and Klug already noted, "viruses could not possibly exist before cells [and] the minimal viruses could be considered highly evolved forms". The delocalized capsid peptide arms may also have a role in the kinetics of the assembly, possibly speeding it up, and it is in this respect intriguing that we clearly see their effects also in the energetics of the assembled viruses.

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