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Virus Isoelectric Point Estimation: Theories and Methods

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ABSTRACT Much of virus fate, both in the environment and in physical/chemical treatment, is dependent on electrostatic interactions. Developing an accurate means of predicting virion isoelectric point (pI) would help to understand and anticipate virus fate and transport, especially for viruses that are not readily propagated in the lab. One simple approach to predicting pI estimates the pH at which the sum of charges from ionizable amino acids in capsid proteins approaches zero. However, predicted pIs based on capsid charges frequently deviate by several pH units from empirically measured pIs. Recently, the discrepancy between empirical and predicted pI was attributed to the electrostatic neutralization of predictable polynucleotide-binding regions (PBRs) of the capsid interior. In this paper, we review models presupposing (i) the influence of the viral polynucleotide on surface charge or (ii) the contribution of only exterior residues to surface charge. We then compare these models to the approach of excluding only PBRs and hypothesize a conceptual electrostatic model that aligns with this approach. The PBR exclusion method outperformed methods based on three-dimensional (3D) structure and accounted for major discrepancies in predicted pIs without adversely affecting pI prediction for a diverse range of viruses. In addition, the PBR exclusion method was determined to be the best available method for predicting virus pI, since (i) PBRs are predicted independently of the impact on pI, (ii) PBR prediction relies on proteome sequences rather than detailed structural models, and (iii) PBR exclusion was successfully demonstrated on a diverse set of viruses. These models apply to nonenveloped viruses only. A similar model for enveloped viruses is complicated by a lack of data on enveloped virus pI, as well as uncertainties regarding the influence of the phospholipid envelope on charge and ion gradients.

KEYWORDS capsid, DNA binding, electrostatic, modeling, polynucleotide, prediction, RNA binding, virion, DNA-binding proteins, RNA-binding proteins, colloid, predictive model, surface charge, virion structure

Electrostatic forces play a critical role in virus fate and transport in engineered and natural systems. Because the charge of organic particles in aqueous solutions is dependent on the ionic environment, notably the hydrogen ion concentration, it is convenient to determine the virus' isoelectric point (pI), i.e., the pH at which the virion's net charge is 0 (neutral), when assessing the probable effects of electrostatic forces. Above their pI, organic macromolecules (such as virions) have a net negative charge due to deprotonated carboxyl groups, while below the pI, protonated amine groups confer a net positive charge. Independent of charge magnitude, knowing even the sign of a viral particle's charge can inform water treatment, such as coagulation (1, 2), disinfection (especially in conditions of virus aggregation) (3, 4), or membrane filtration (5), as well as modeling virus transport through porous media (6) and virus sampling and concentration (7–10).

Electrostatic forces are not the sole determiner of virus fate and transport; other interactions, such as van der Waals forces, the hydrophobic effect, cation bridging, and steric interactions, also play a prominent role in virus interactions with the surrounding

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environment (1, 5, 11). In turn, *pI* is not a perfect indicator of electrostatic forces under all conditions. Electrophoretic mobility away from the *pI* is highly dependent on environmental conditions, e.g., conductivity (5). The *pI* cannot indicate whether a given virus may alternate definitively between strong positive and negative surface charges below and above the *pI* or meander near zero charge over a broad pH range. While electrostatic forces are not a perfect predictor of virus physical/chemical interactions, and *pI* is not a perfect indicator of electrostatic forces under all conditions, *pI* provides a reliable and quantitative benchmark for comparing environmental interactions of different viruses across a range of conditions and experimental methods. In addition, focusing on *pI* allows us to address the greatest disparities between theoretical and empirical results before proposing a more refined model to estimate the magnitudes of surface charge and potential.

Many attempts have been made to model the *pI* of nonenveloped viruses based on ionizable residues within capsid proteins (12–16). However, major discrepancies arise between predicted *pIs* based on capsid proteins and empirically determined virus *pIs*. While empirical *pIs* are commonly reported in the acidic range (pH 2 to 5) (17), capsid proteome sequences overwhelmingly contain balanced concentrations of amino acids reflecting predicted *pIs* near neutral (pH 5.5 to 8) (18, 19). Therefore, capsid amino acid composition alone cannot account for virus *pI*.

Several researchers have proposed electrostatic models of the virion to explain the poor predictive value of ionizable amino acids. Based on a “soft colloid” model proposed by Duval and Ohshima (20), Langlet et al. (21, 22) and Dika et al. (5, 23) suggested that nucleic acids at the core of the virus capsid contribute to overall virus surface charge. Schaldach et al. (16) developed a similar permeable virion model that weighted the influence of capsid moieties based on electrostatic screening of the surrounding medium. Both models suggest that with increasing permeability, buried components of the virion have a greater impact on the overall *pI* (16, 21, 23). In contrast, Penrod et al. (15) and Armanious et al. (13) suggested that only exterior residues contribute to the surface charge; thus, heterogeneous distribution of positive and negative amino acid charges within the capsid coat results in higher or lower *pI* values. Božič et al. (14) also evaluated a one- or two-shell model of virion surface charge to account for heterogeneity in ionizable amino acid distribution, though the model was specifically applied to empirical *pI* values only for bacteriophage PP7 (24). While the debate around fundamental hypotheses can be polarizing, not all elements of these models are contradictory.

Recently, Heffron and Mayer (25) suggested a divergent approach to modeling non-enveloped virion *pI* based not on a single electrostatic model of the virion but rather on the variable extent of electrostatic interactions between the capsid and the viral genome. Since several of the viruses with the greatest discrepancy between predicted and empirical *pIs* featured large capsid regions devoted to binding the viral polynucleotide, Heffron and Mayer hypothesized that the charges of these polynucleotide-binding regions (PBRs) and bound sections of the viral polynucleotide itself are mutually neutralized. The authors also predicted the location of PBRs from virus capsid proteome sequences to predict the *pI* of viruses whose detailed capsid structures were unknown. This approach supported the observations of Šiber and Podgornik (26) that the two-shell model of Božič et al. (14) was appropriate for spontaneously assembling viruses with strong, nonspecific interactions between capsid proteins and single-stranded RNA (ssRNA). However, the PBR exclusion approach showed improvement in *pI* prediction for double-stranded DNA (dsDNA) viruses as well as ssRNA viruses (25).

The goal of this review was to evaluate the potential of polynucleotide influence and exterior residue theories for developing a model of nonenveloped, icosahedral virus *pIs*, compared to the newly proposed hypothesis of PBR exclusion. In “Polynucleotide charge contribution,” models suggesting polynucleotide influence are discussed in light of empirical evidence. In “Surface-weighted capsid models,” the theory that external capsid residues contribute disproportionately to overall charge is investigated using three-dimensional (3D) capsid structures for 26 viruses with known (empirical) *pIs*. In “Polynucleotide-binding

TABLE 1 Studies comparing pls of VLPs and whole virions^a

Virus species	Virus pl	VLP pl	Theoretical VLP pl ^b	Concn/purification method	VLP method	VLP quality control	Reference
Adeno-associated virus 4	2.6	2.6	5.8	Centrifugation, dialysis	Naturally occurring	Differential sedimentation	Salo and Mayor, 1978 (102)
Enterobacteria phage MS2	3.9	4.0	7.4	Chloroform lysis, centrifugal filtration	<i>In situ</i> degradation	Fluorometric RNA assay	Armanious et al., 2016 (13)
Enterobacteria phage MS2	3.5	3.4–3.8	7.4	Centrifugation, dialysis	Clonal plasmid expression	Electron microscopy	Dika et al., 2011 (23)
Enterobacteria phage MS2	3.3	3.3	7.4	PEG precipitation	<i>In situ</i> degradation	Electron microscopy	Nguyen et al., 2011 (103)
Feline panleukopenia virus	5.0–5.3	5.3	5.4	PEG precipitation, ultracentrifugation, dialysis	Unknown	Differential sedimentation	Weichert et al., 1998 (104)

^aVLP, virus-like particle (capsid lacking the viral genome).

^bTheoretical VLP pl calculated here is based on the total charge of ionizable amino acids in capsid proteins.

regions,” Heffron and Mayer’s approach of excluding PBRs is discussed, as is the model of virion charge structure that arises from the PBR exclusion approach. Finally, the potential of these competing theories for developing a predictive pl model is discussed in “Further considerations for a predictive isoelectric point model,” as are the impediments to applying such a model to enveloped viruses.

POLYNUCLEOTIDE CHARGE CONTRIBUTION

Some researchers (16, 21, 23, 27) have accounted for the differences between theoretical and empirical virion pl by developing models of virions as permeable colloids. A permeable virion implies that interior charges can affect overall virion charge. The main, hypothesized interior charge contribution comes from the densely packaged polynucleotide core. Polynucleotide phosphodiester groups have a pK_a near pH 1 and would therefore contribute a negative charge even at very low pH. Below approximately pH 5, this charge would be moderated by positive charges from amino groups on adenine, cytosine, and guanine (28). Still, the overall charge of the polynucleotide would be net negative at $pH > 1$, since all nucleotides have a phosphate group, regardless of base.

However, the polynucleotide folding necessary for virion packaging is mediated by a cloud of counterions that to some degree negates electrostatic repulsion (29–32). While many of these counterions may be released in mature virions (33), the viral core likely retains a relatively high concentration of divalent cations (34). Thus, the presence of an overwhelming negative charge at the virion core is not a foregone conclusion. If the nucleic acid core impacts virion charge, the effect should be empirically demonstrable via (i) comparison of whole virions to virus-like particles (VLPs) and/or (ii) comparison of virion pl in various ionic strength solutions.

VLPs are viral capsids lacking all or most of the internal genome. As shown in Table 1, VLPs have pls extremely similar to those of the corresponding intact virus, even when the predicted pl differs greatly. In interpreting these results, Dika et al. (23, 35) hypothesized that some negatively charged host material was trapped within the VLPs during propagation or that virus purification by polyethylene glycol (PEG) precipitation may coat the capsid surface and cause insensitivity to charge contributions from the virion core. However, the comparatively empty state of VLPs was confirmed in most experiments by a variety of quality control methods, as listed in Table 1. A demonstrably lower core content would be expected to have some impact on pl. Yet, VLPs had the same pl as whole virions in tests using a variety of protocols for both purification and assessment of polynucleotide content of VLPs. In their study comparing purification protocols, Dika et al. (35) also did not use a solvent extraction phase (e.g., washing with chloroform or Vertrel), which typically follows the concentration phase to remove the PEG and promote monodispersion (1). To accept the hypothesis that the viral

polynucleotide influences pI , we should see a distinct increase in the pI of VLPs compared to that of whole virions, which was not observed.

The influence of the polynucleotide on virion charge should also be evident by tests at various ionic strengths. If capsids are permeable, electrostatic screening due to the electrolyte solution should influence the distance from the exterior surface at which buried charges can influence the surface charge. Since Debye lengths in freshwater are on the order of 10 to 100 Å (36), and virus capsids are typically 20 to 40 Å thick (14), the virion components contributing to surface charge could be expected to vary depending on ionic strength. The impact of electrolyte screening on depth of charge influence was the hypothesis behind the modeling approach used by Schaldach et al. (16), which weights the influence of ionizable amino acids based on depth within the capsid as a function of solution ionic strength, I . Thus, capsid residues are progressively weighted based on their proximity to the exterior capsid surface. For simplicity, the virion is typically modeled as a perfect sphere, with the exterior surface defined as the outer radius, as shown in Fig. 1C. However, this simplification is likely not appropriate for viruses with a high degree of crenulation. The Schaldach et al. model (16) also assumes a negatively charged virion core. Results closely matched electrophoretic mobility measurements of bacteriophage MS2 and norovirus VLPs at an I of 0.01 M and bacteriophage Q β in solution with an I of 0.1 M. However, the team determined that the absence of polynucleotide influence did not impact the fit of the norovirus model to empirical data using VLPs. Nap et al. (24) used a contrasting model developed by Božič et al. (14) for dividing capsids into inner and outer shells to explain the impact of I on the pI of bacteriophage PP7 as reported by Brorson et al. (8) (see Table 2).

Many investigators (8, 22, 37, 38) have measured variations in pI over various ionic strengths. Table 2 provides a summary of experiments in which a single researcher evaluated virus pI at multiple ionic strengths. To support the hypothesis that the core polynucleotide contributes significantly to overall capsid charge, measured pI should increase at higher ionic strength. Overall, however, the pI s did not increase uniformly with I to reflect a substantial charge contribution from the core, and changes were not on the scale expected from the difference in pI of capsid proteins ($pI \sim 5.5$ to 8) and nucleic acids ($pI \sim 1$) (18, 28). Even when I varied by 2 orders of magnitude (Debye lengths from ~ 10 nm to 1 nm), these dramatic differences were not seen between pI s. Rather, virus pI s increased, decreased, or remained constant with increasing I , indicating a virus-specific response expected from heterogeneous charge distributions.

The precision of pI measurements decreases with increasing I due to reduction of surface charge from electrostatic shielding (39). Therefore, even the minor variation observed in pI s at low and high I could be explained by this lack of precision. For the empirical pI s referenced in Michen and Graule's review (17), I ranged from ~ 0.5 mM, typical of isoelectric focusing in ampholyte buffers (40), to ≥ 100 mM in concentrated electrolyte solutions (22, 38, 41–43). In addition, viruses are more likely to aggregate at high ionic strength, while the models discussed here assume monodispersion. Differences in I and electrolyte composition are likely responsible for some variation in reported pI s (17). Nonetheless, there remains broad agreement between empirical pI s where multiple experiments are available, despite widely differing solution compositions and measurement techniques (17).

Overall, empirical evidence does not support the hypothesis that the polynucleotide contributes strongly to net virion charge. This lesser contribution may be due to counterions retained within the capsid (34), resulting in a lesser negative-charge magnitude. The ionic composition of the virion core may play a major role in overall virion charge. However, more research is needed to determine the composition and impact of counterions around the polynucleotide. The main support for polynucleotide charge contribution comes from theoretical models to account for the discrepancy between theoretical and empirical pI s of *Leviviridae* phages (21–23). However, *Leviviridae* phages have distinctive thin capsids with large, positively charged interior regions devoted to polynucleotide binding (44–46) and are therefore poor exemplars of virion structure.

FIG 1 Impact of including only exterior residues in predicted pI calculation using 3D capsid structures. (A to C) The mean empirical pI value for each unique virus is shown in comparison to the virus' predicted pI calculated from exterior capsid residues. Exterior residues represent increasingly narrow shells determined by fraction of outermost amino acids (A) and distance from the exterior surface (B), as illustrated in panel C. Distances in panel B are displayed on a log color scale to clearly show the impact on all viruses despite large disparities in capsid size and thickness. In both panels A and B, a lighter tint indicates a narrower "slice" of the capsid, while a darker tint indicates that a larger portion of the capsid was considered in the pI calculation. The diagonal line represents equivalent theoretical and empirical pIs; to accept either method of calculating pI based on exterior residues, points of similar tint should be clustered along this line. Two groups that appeared to benefit most from including only exterior residues are labeled in the figure: ssRNA *Leviviridae* phages with basic, interior beta sheets (bacteriophages fr [EBFR], GA [EBGA], MS2 [EBMS2], and Q β [EBQB]) and ssRNA viruses with basic, interior N termini (cowpea chlorotic mosaic virus [CCMV], cucumber mosaic virus [CMV], red clover necrotic mosaic virus [RCNM], and southern bean mosaic virus [SBMV]).

SURFACE-WEIGHTED CAPSID MODELS

Some electrostatic models have relied on detailed 3D virus structures to attempt pI prediction, either instead of or in addition to supposing a polynucleotide charge contribution. Presuming an impermeable capsid, Penrod et al. (15) accounted for the measured pI of enterobacteria phage MS2 by evaluating only those charged structures exposed on the surface of the capsid. Armanious et al. (13) also successfully employed this method to account for the pI of MS2 and three other bacteriophages of the family

TABLE 2 Studies comparing pls at various ionic strengths^a

Reference and virus	Capsid outer radius ^b (nm) (reference)	Measured pI	Ionic strength, I (mM)	Estimated Debye length ^c (nm)	Electrolyte	Method ^d
Brorson et al., 2008 (8)					NaCl plus buffer	CF
Enterobacteria phage PP7	15	4.9	<1	>9.6		
		4.7	40	1.5		
		4.3	100	1.0		
Enterobacteria phage PR772	~29 (105)	4.4	<1	>9.6		
		4.3	5	4.3		
		4.3	20	2.2		
		4.2	40	1.5		
Enterobacteria phage ΦX174	17	7	<1	>9.6		
		7.3	5	4.3		
		7.5	10	3.1		
		7.8	20	2.2		
Langlet et al., 2008 (22)					NaNO ₃	EM
Enterobacteria phage GA	14	2.1	1	9.6		
		2.3	100	1.0		
Enterobacteria phage MS2	14	3.1	1	9.6		
		3.9	100	1.0		
Enterobacteria phage Qβ	15	2.7	1	9.6		
		1.9	100	1.0		
Enterobacteria phage SP	~15 ^e	2.1	1	9.6		
		2.6	100	1.0		
Molodkina et al., 1986 (37)					NaCl	EM
Influenza A virus H1N1	~50 (106)	4.5	0.2	21		
		4.35	0.4	15		
		4.25	2	6.8		
		4	10	3.1		
Taylor and Bosmann, 1981 (38)					NaCl	EM
Mammalian orthoreovirus 3	43	3.8	1	9.6		
		3.8	10	3.1		
		3.8	100	1.0		

^aSummarized from a report by Michen and Graule (17).
^bOuter radius values were obtained from the ViperDB database (107), except as noted.
^cDebye length in 1:1 electrolyte approximated by the formula Debye length (nm) $\approx 0.305(I (M))^{-1/2}$, as described by Otterstedt and Brandreth (108).
^dCF, chromatofocusing; EM, electrophoretic mobility.
^eApproximate radius based on Qβ, which is in the same genus (*Allolevivirus*) and shares 80% similarity in coat protein amino acid sequence (109).

Leviviridae. Many other viruses with acidic pls feature a concentration of basic amino acids toward the capsid interior. Therefore, the exclusion of interior residues decreases the predicted pI of these viruses and may better approximate some acidic empirical pls. As further discussed in “Polynucleotide-binding regions,” this concentration of basic residues is not applicable to all viruses. Božič et al. (14) noted that this concentration of basic charges within virus capsids defied any simple pattern or classification based on virus structure. The asymmetrical distribution of capsid charges was instead attributed to nonspecific electrostatic interactions involved in virus self-assembly (26). In addition, experimental evidence contradicts the theory of a capsid that is completely impermeable to electrolytes (16). From a practical perspective, not only is manually selecting exterior-exposed residues labor-intensive, but the definition of “capsid exterior” also begins to blur for thicker capsids with extensive crenulations. Given the subjectivity and tedium of manually selecting individual capsid residues, a method to separate the capsid into exterior (charge-contributing) and interior (noncontributing) shells would be beneficial. Božič et al. (14) suggested that such a model could be applied for some viruses, though many viruses do not show a two-shell charge distribution. The applicability of the two-shell model was later determined to be dependent on interactions between capsid and polynucleotide (47). Thus, Božič et al. did not use the two-shell model to predict empirical pls for a range of viruses.

Based on a set of 26 viruses with available detailed 3D structures and empirical pI values, we attempted to define an interior and exterior shell based on (i) relative

distribution within the capsid and (ii) absolute distance from the capsid surface. (Further details of this analysis can be found in the supplemental material [Section S1, “Methods for figure generation”].) As shown in Fig. 1, inclusion of only exterior residues did not improve pI prediction for all viruses, regardless of how exterior residues were defined. (These data are also presented in detail in Fig. S1 in the supplemental material for the entire range of exterior fractions and distances.) Of the viruses evaluated, two groups appeared to benefit most from including only exterior residues: ssRNA *Leviviridae* phages with basic, interior beta sheets (bacteriophages ϕ [EBFR], GA [EBGA], MS2 [EBMS2], and Q β [EBQB]) and ssRNA viruses with basic, interior N termini (cowpea chlorotic mosaic virus [CCMV], cucumber mosaic virus [CMV], red clover necrotic mosaic virus [RCNM], and southern bean mosaic virus [SBMV]), as labeled in Fig. 1. However, no one method of slicing the capsid was optimal even for these eight viruses. For the remaining viruses, predicted pI was slightly more likely to trend away from the range of empirical pIs when only exterior residues were considered (Fig. S1). Therefore, the strategy of selecting only exterior residues cannot be used indiscriminately to predict unknown pIs.

Schalldach et al. (16) used a more nuanced 3D model, in which electrostatic screening was modeled by inversely weighting residues by distance from the capsid surface as a function of Debye length. By this model, calculating theoretical charge with all capsid residues better matched empirical electrophoretic mobility measurements than using surface residues only. However, the Schalldach model required a theoretical polynucleotide charge to account for the charge of *Leviviridae* phages MS2 and Q β , whereas the polynucleotide influence was irrelevant to the norovirus model (16).

Heffron and Mayer (25) determined that removing only the interior surface-exposed residues resulted in the best fit between theoretical and empirical pIs for a diverse set of 21 viruses, whereas models using only the exterior surface residues showed no apparent correlation to overall capsid pI. The interior surface of many viruses is involved in viral polynucleotide binding (48–52). Because capsid surfaces are irregular, identifying and removing interior-accessible residues would most selectively remove structures like the interior beta sheets of *Leviviridae* and arginine-rich regions in the disordered N termini of many ssRNA plant viruses, two groups that benefitted greatly from excluding interior residues (Fig. 1). As further discussed in “Polynucleotide-binding regions,” these basic, interior capsid features are noncovalently bound to the viral RNA (46, 48, 52, 53), and therefore their positive contribution to virion charge is likely negated by the negatively charged polynucleotide.

Some authors (13) have also excluded from pI calculation residues whose surface area is buried by the folding of the polypeptide. Practically, buried residues may be defined as having a relative solvent-accessible surface area less than 20% that of the corresponding amino acid, based on models with an approximate resolution of 3 Å (13). Using this cutoff did not improve the overall pI prediction for the whole capsid or outer capsid residues (Fig. S2) nor did attempts to weight amino acid influence by solvent-accessible surface area alone or in combination with relative or absolute distance from the exterior (data not shown). Excluding buried residues selects against beta sheets, the least solvent-accessible protein structures (54). Ignoring beta sheets may work well for *Leviviridae* phages, in which most large beta sheets are involved in RNA binding (46). However, beta sheets are major components of many virus capsids (e.g., “jelly roll” folds) and should not be discounted from charge calculations without strong justification.

POLYNUCLEOTIDE-BINDING REGIONS

PBRs are a feature of many virus capsids. While some viruses (e.g., many dsDNA viruses and picornaviruses) feature genomes that are covalently bound, often to a single, small capsid protein (55–58), many virus polynucleotides are noncovalently bound via electrostatic interactions with residues on the capsid interior (PBRs). The two methods of polynucleotide binding reflect different packaging strategies; dsDNA and dsRNA

polynucleotides are typically spooled into a previously formed capsid, while capsids that are formed spontaneously by assembly of subunits require more extensive bonding between the polynucleotide and capsid (34, 44, 59). PBRs may occur in a single region of a capsid protein sequence, as in the disordered, arginine-rich terminal domains in many positive-stranded RNA viruses (e.g., the ssRNA plant viruses mentioned in “Surface-weighted capsid models”) (52, 59–62) or along broader, positively charged regions (“clefts”) that are contiguous on the protein surface but not necessarily continuous in the primary sequence (e.g., negative-stranded ssRNA viruses and *Leviviridae* phages) (46, 63). In either case, the predominantly basic charges of the interior capsid PBR residues would be countered via this electrostatic interaction with the polynucleotide. The polynucleotide segment and PBR would therefore not contribute to overall virion charge, and these regions should be excluded from theoretical charge calculations.

Heffron and Mayer (25) evaluated the effect of excluding predicted PBR regions from capsid charge calculations and reported an overall improvement in accuracy of the modified pI predictions compared to the unmodified predictions, from a deviation of 2.1 ± 2.4 to 0.1 ± 1.7 pH units. This difference was significant to a high degree of confidence ($P = 4 \times 10^{-8}$) (25). (A list of the viruses evaluated in this study is provided in Table 3.) A comparison of capsid charge predictions with and without PBRs is shown in Fig. 2, based on the empirical pIs and predicted PBRs presented by Heffron and Mayer (25). (Section S1 details how this plot was generated.) Compared to the original predictions without modification (Fig. 2A), far more empirical pIs fall within the range of theoretical net-neutral charge after modification (Fig. 2B). Predicting pH ranges of low net charge (as visualized here) may be more valuable than a single, predicted pI, as the virion may function similarly over a pH region around the pI, including in behaviors relied on for empirical pI measurements (e.g., aggregation and electrophoresis). Although Heffron and Mayer did not advance a model for surface charge magnitude, the analysis in Fig. 2 provides a qualitative account of how surface charge varies with pH. Knowing the breadth of these regions is valuable for predicting the effect of pH on phenomena such as aggregation and surface adhesion.

Although ssRNA viruses showed some of the greatest improvements from PBR exclusion in the report of Heffron and Mayer (25), the need for PBR exclusion was not predictable based on genome type. Several enteroviruses showed slightly poorer pI prediction after PBR exclusion, despite having ssRNA genomes. This result can be seen for the enteroviruses poliovirus 1 (POL1), coxsackieviruses A21 and B5 (CXA21, CXB5), human rhinovirus 2 (HRV2), and echovirus 1 (ECV1), as well as the closely related Mengo encephalomyocarditis virus (MEV) (Fig. 2). Enteroviruses and other picornaviruses form capsids primarily through protein-protein binding, rather than protein-RNA binding (64), and thus provide an example of ssRNA viruses with no need for PBR exclusion. Furthermore, the dsDNA viruses human adenovirus 5 (HAdV5) and cottontail rabbit papillomavirus (CRPV) showed notable improvement after PBR exclusion. HAdV5 contains histone-like proteins and core proteins for dsDNA packaging (65–67), while papillomavirus major and minor capsid proteins have DNA-binding C and N termini, respectively (68, 69). All of these regions were identified as arginine-rich regions via PBR prediction in the report of Heffron and Mayer (25). Therefore, the usefulness of the PBR exclusion method is not restricted to only ssRNA viruses. Greater specificity in PBR prediction will likely also improve pI prediction for a wider range of viruses.

To summarize the picture of virion charges developed thus far, (i) the polynucleotide does not show evidence of contributing a strong negative charge and may be coulombically neutralized by a cloud of counterions (34) and (ii) the best 3D model that does not suppose polynucleotide influence suggests that only charges on the interior surface should be omitted, regardless of capsid dimensions. Omitting interior surface charges showed the greatest improvement in pI prediction for ssRNA viruses with interior concentrations of basic residues devoted to polynucleotide binding. If the unbound polynucleotide retains a cloud of counterions after folding and packaging, the impact of the polynucleotide on overall virion charge would be observed primarily as neutralization of

TABLE 3 Classification and abbreviations for viruses^a

Abbreviation	Species	Genus	Nucleic acid	NCBI taxon ^b	PDB ID ^c (reference)	Resolution (Å)
AAV4	Adeno-associated virus 4	<i>Dependoparvovirus</i>	ssDNA	57579	2G8G (110)	3.2
BDMV	Belladonna mottle virus	<i>Tymovirus</i>	ssRNA	12149		
BP29	<i>Bacillus</i> phage Φ29	<i>Salasvirus</i>	dsDNA	10756		
CCMV	Cowpea chlorotic mottle virus	<i>Bromovirus</i>	ssRNA	12303	1CWP (111)	3.2
CMV	Cucumber mosaic virus	<i>Cucumovirus</i>	ssRNA	12307	1F15 (112)	3.2
CPaV2	Canine parvovirus 2	<i>Protoparvovirus</i>	ssDNA	10790		
CPaV2 ^d	Feline panleukopenia virus	<i>Protoparvovirus</i>	ssDNA	10787	1C8G (113)	3.0
CRPV	Cottontail rabbit papillomavirus	<i>Kappapapillomavirus</i>	dsDNA	31553		
CRPV ^d	Human papillomavirus 16	<i>Alphapapillomavirus</i>	dsDNA	333760	5KEQ (114)	4.3
CXA21	Coxsackievirus A21	<i>Enterovirus</i>	ssRNA	12070	1Z7S (115)	3.2
CXB5	Human coxsackievirus B5	<i>Enterovirus</i>	ssRNA	103907		
CXB5 ^d	Human coxsackievirus B3	<i>Enterovirus</i>	ssRNA	103904	1COV (116)	3.5
EBFR	Enterobacteria phage fr	<i>Levivirus</i>	ssRNA	12017	1FRS (117)	3.5
EBGA	Enterobacteria phage GA	<i>Levivirus</i>	ssRNA	12018	1GAV (118)	3.4
EBMS2	Enterobacteria phage MS2	<i>Levivirus</i>	ssRNA	329852	2MS2 (119)	2.8
EBQB	Enterobacteria phage Qβ	<i>Allolevivirus</i>	ssRNA	39803	5VLY (120)	3.3
EBSP	Enterobacteria phage SP	<i>Allolevivirus</i>	ssRNA	12027		
ECV1	Echovirus 1	<i>Enterovirus</i>	ssRNA	103908	1EV1 (121)	3.6
ELV	Erysimum latent virus	<i>Tymovirus</i>	ssRNA	12152		
HAdV5	Human adenovirus 5	<i>Mastadenovirus</i>	dsDNA	28285	4V4U (122)	10
HHAV	Hepatitis A virus	<i>Hepatovirus</i>	ssRNA	12098	4QPI (123)	3.0
HRV2	Human rhinovirus 2	<i>Enterovirus</i>	ssRNA	12130	1FPN (124)	2.6
MEV	Mengo encephalomyocarditis virus	<i>Cardiovirus</i>	ssRNA	12107	2MEV (125)	3.0
NOR1	Norwalk virus	<i>Norovirus</i>	ssRNA	524364	1IHM (126)	3.4
PHIX	Enterobacteria phage ΦX174	<i>Sinheimvirus</i>	ssDNA	10847	2BPA (127)	3.0
PM2	Pseudoalteromonas phage PM2	<i>Corticovirus</i>	dsDNA	10661	2W0C (75)	7.0
POL1	Poliovirus	<i>Enterovirus</i>	ssRNA	12081	1HXS (128)	2.2
PRD1	Enterobacteria phage PRD1	<i>Alphatectivirus</i>	dsDNA	10658	1W8X (76)	4.2
RCNM	Red clover necrotic mosaic virus	<i>Dianthovirus</i>	ssRNA	12267	6MRM (129)	2.9
REO3	Reovirus 3	<i>Orthoreovirus</i>	dsRNA	10886	2CSE (130)	7.0
SBMV	Southern bean mosaic virus	<i>Sobemovirus</i>	ssRNA	652938	4SBV (131)	2.8
ScrMV	Scrophularia mottle virus	<i>Tymovirus</i>	ssRNA	312273		
SRVA	Simian rotavirus A	<i>Rotavirus</i>	dsRNA	450149	4V7Q (132)	3.8
TBMV	Tobacco mosaic virus	<i>Tobamovirus</i>	ssRNA	12243		
TYMV	Turnip yellow mosaic virus	<i>Tymovirus</i>	ssRNA	12154	1AUY (133)	3.0

^aAs previously used by Heffron and Mayer (25).

^bNCBI Taxon, National Center for Biotechnology Information (<https://www.ncbi.nlm.nih.gov>) taxonomical ID (134).

^cPDB ID, Protein Data Bank (<https://rcsb.org>) ID used for 3D structural comparisons (135).

^dAlternate species/strain used for 3D structure only.

these PBR charges. Finally, attempts to predict pl by excluding known and predicted PBR regions showed significant improvement in pl prediction for a wide range of viruses (25). Figure 3 presents a hypothesized conceptual model of virion charges that arises from the PBR exclusion approach. This conceptual model does not account for either (i) quantitative charge (except net-neutral charge at the pl) or (ii) nonelectrostatic forces within the virion, such as osmotic pressure and polymer elasticity (47). However, we hope this model can serve as a basis for further conversation and refinements.

FURTHER CONSIDERATIONS FOR A PREDICTIVE ISOELECTRIC POINT MODEL

Previous capsid charge models have been based on theoretically provable assumptions of polynucleotide charge contribution or capsid impermeability/nonpermeability. As discussed in “Surface-weighted capsid models” and “Polynucleotide-binding regions,” empirical evidence tends to contradict the core principles behind both of these assumptions. Regardless of the reality of virion charge structure, these two approaches to calculating theoretical virion pl have a serious practical impediment to developing a predictive pl model, in that both must be fit to empirical pl data. A polynucleotide contribution model must determine the extent of core contribution, as well as account for differences in capsid size, geometry, and apparent importance of polynucleotide influence (e.g., between leviviruses and enteroviruses). A surface residue-only model requires a universal

FIG 2 Theoretical average charge of virus proteomes before (A) and after (B) modification by removing predicted polynucleotide-binding regions, as reported by Heffron and Mayer (25). Empirical pI values from the literature are shown as purple circles. Good fit between theoretical and empirical pIs is indicated when the purple circles fall within the white space (net-neutral virion surface charge) of the colored bars. Theoretical charge was calculated based on the sum of ionizable amino acids in capsid proteome sequences. Highly represented virus families (>2 representatives) are noted by letters to the right of each graph: L, *Leviviridae*; P, *Picornaviridae*; and T, *Tymoviridae*. A key to the virus abbreviations (y axis) is provided in Table 3.

criterion for defining surface residues for capsids of various sizes and structures. Pending a dramatic push to expand and verify virus pI data, current empirical pIs are few, poorly corroborated, and overrepresentative of a few virus genera (e.g., *Levivirus*, *Enterovirus*, and *Tymovirus*) (17, 25).

The overrepresentation of *Leviviridae* in the literature is a particular problem, as these ssRNA bacteriophages have been the exceptions around which models were built (13, 15, 22, 70). A large amount of the interior capsid surface of *Leviviridae* phages is devoted to polynucleotide binding (~57% of the MS2 capsid protein) (44–46). The predicted pI of these phages can be brought into accordance with empirical pI by (i) excluding these predominantly basic residues on the basis of capsid impermeability or polynucleotide binding or (ii) proposing a strong negative charge from the virion core. However, the same model must also be applicable to a wide range of viruses that do not share these features.

Among these approaches, the PBR exclusion method is unique in that it is nonarbitrary, i.e., the inclusion or exclusion of a residue in the charge calculation is predicted according to an independent criterion (whether or not a residue occurs in a PBR), rather than directly from the impact of that residue on charge. No additional fitting is required to translate the predicted PBR into a weighted charge contribution; predicted PBR residues are simply excluded. Therefore, PBR exclusion is less likely than previous models to overfit pI prediction to the limited empirical data available. The PBR prediction method outlined by Heffron and Mayer (25) also relied on proteome sequences

FIG 3 Hypothesized electrostatic model of the virion including polynucleotide-binding regions. Capsid proteins as a whole contain a balance of acidic and basic residues. At a given pH, these residues range across a broad spectrum of charge from strongly negative (dark red), to neutral (white), to strongly positive (dark blue). However, some viruses have a high concentration of basic residues on the capsid interior which are electrostatically bound to the polynucleotide. The charges of both the polynucleotide-binding regions of the capsid and associated polynucleotide segments are mutually negated. The charge of the polynucleotide core is screened by a hypothesized cloud of counterions retained in the virion core. The overall charge arises from the nonbinding portions of the capsid, which have an acidic pI due to a disproportionately low concentration of basic residues.

alone, obviating the need for detailed 3D capsid models, another research bottleneck. In addition, neither polynucleotide influence nor impermeable colloid models have been applied to a wide range of diverse viruses. When applied to a diverse set of viruses, the PBR exclusion approach accounted for very acidic pIs without sacrificing prediction of circumneutral pIs for viruses lacking PBRs.

The PBR exclusion method also reconciles the lack of empirical evidence for a strong charge contribution from the capsid interior. After PBR exclusion, differences between empirical and predicted pIs for the virus set evaluated by Heffron and Mayer (25) were distributed around a mean of 0.1, whereas viruses without modification had predicted pIs on average 2.1 pH units higher than empirical values. Therefore, the PBR exclusion method agreed with empirical data suggesting at most a minor charge contribution from the polynucleotide, as discussed in "Polynucleotide charge contribution." When comparing pIs in solutions of various ionic strengths, the extremely basic regions neutralized via nucleotide binding would not impact virion charge, regardless of *I* and location within the capsid structure.

Also, PBRs may be neutralized even in VLPs. As suggested by Dika et al. (23), VLPs may retain some nucleic acid or host cell material (23, 71). Since electrostatic PBR-polynucleotide interactions are nonspecific (26, 52), the extent of capsid charge largely determines the amount of encapsidated material (59). However, ssRNA viruses typically contain roughly twice the charge equivalent of polynucleotide compared to PBR charge (59, 72). If the polynucleotide influenced overall virion charge, the decreased density of the VLP core should impact overall virion charge, whereas a lesser amount of material may be sufficient to neutralize PBRs. For viruses that rely on electrostatic interactions for assembly, inclusion of a threshold of negatively charged material may be required for intact VLPs.

While the PBR exclusion method somewhat vindicates the approach of negating interior residues, this approach was beneficial only for viruses with substantial PBRs on the capsid interior. A few predictions were dramatically worse when removing interior residues (Fig. 1 and Fig. S1). Even for bacteriophage GA (EBGA), a levivirus with RNA-binding beta sheets, most methods of defining exterior residues resulted in a prediction several pH units from the empirical pI (Fig. S1). Therefore, a physical model that incorporates the permeable and charge-permitting nature of the capsid appears more valid than an impermeable capsid model. However, such a model should build from the insights in the study of Heffron and Mayer (25) showing that the charge contribution of PBRs should be excluded. This avoids imposing arbitrary variables for a negative core charge or virion permeability without experimental support.

Considerations for enveloped viruses. Current models of virion charge are limited to nonenveloped, icosahedral virions. Environmental persistence of viruses with phospholipid envelopes, such as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is generally considered insufficient to be relevant to transport or water and wastewater treatment (73, 74). For this reason, only nonenveloped viruses were considered here, except for bacteriophages PM2 and PRD1, which contain an internal lipid membrane (75, 76). However, some enveloped viruses, especially those transmitted via the fecal-oral route (e.g., avian influenza virus), may persist for months in aqueous environments (77, 78). In addition, the electrostatic charge of enveloped viruses may inform virus removal via air filtration and deposition on surfaces.

Unfortunately, enveloped viruses present unique challenges to pI prediction. Envelope phospholipids may contribute substantially to surface charge, and the low dielectric constant of phospholipid bilayers may decrease their apparent pK_a by as much as one pH unit (79). The diversity of phospholipids in virus envelopes may also defy efforts for a predictive model. Ivanova et al. (80) quantified over 125 different phospholipids from three strains of influenza virus and found that the composition of lipids in the virion envelopes differed not only from the host cell membrane but also between virus strains. Since these phospholipids are acquired from the host, the complex lipid profiles are not predictable from the viral genome. Virions may acquire other materials from the host as well. For example, human papillomavirus acquires histones from the host that stabilize the polynucleotide within the capsid (81). These structures are likewise not coded for in the viral genome yet may impact overall capsid charge by neutralizing the polynucleotide charge.

Perhaps most damning is that empirical pI data for enveloped viruses are particularly sparse, with poor agreement between sources, as summarized in Fig. 4. Unfortunately, only three genera are represented in Michen and Graule's exhaustive review of empirical pI data (17), although pIs of isolated proteins (and especially glycoproteins) from enveloped viruses are more common (82–86). Empirical pIs for several strains of *Orthopoxvirus* are available, but much of the data come from two research groups with poor agreement, even when the same virus strains are being compared (87). As previously observed for nonenveloped viruses (25), pI measurements based on electrophoretic mobility were more acidic than measurements made by isoelectric focusing or other methods. However, the method of measurement was confounded by the source. Douglas et al. (88, 89) performed the majority of enveloped virus electrophoretic mobility measurements, whereas Mouillot and Netter (90) were responsible for the majority of isoelectric focusing measurements (17, 87). Douglas et al. used a more rigorous purification process than Mouillot and Netter (17). However, Douglas et al. performed experiments in molar sucrose, which may have impacted virion charge, aggregation, and electrophoretic mobility (89). Therefore, it is difficult to determine if there is a true difference between the two methods. In addition, poxviruses may have multiple infectious forms and numerous membrane-embedded proteins (91). For development of a theory of enveloped virus pI, the priority should be collecting empirical pI values for strains of viruses with one or two well-defined membrane proteins (e.g., coronaviruses or influenza A virus [92, 93]). However, the wide diversity in

FIG 4 Distribution of empirical pI values for enveloped viruses referenced by Michen and Graule (17). Box plots summarizing the pIs for each virus genus are overlaid with individual pI values. Individual pI values are distinguished by method of determination (color) and literature source (shape). Two teams, Douglas et al. (88, 89) and Mouillot and Netter (90), were responsible for all *Orthopoxvirus* empirical pIs in this plot; all other sources (37, 98–101) are labeled in the figure. Points are horizontally scattered within each group for clarity only.

envelope proteins between strains may still present a challenge to extrapolation of a model to novel viruses.

Interactions between viruses and the surrounding environment. Ions in the water matrix may bind to moieties on the capsid surface, thereby altering surface charge. This is especially true of multivalent ions such as calcium and phosphate (17, 38), which may even be retained after viruses are transferred from the propagation/storage solution (6). In addition to ions from the surrounding medium, polyvalent cations are integral to the structure of many viruses. These ions may significantly alter pI and may be so integral as to be removed only through denaturation (94). Of the viruses shown in Fig. 2, five viruses (*Bacillus* phage Φ 29 [BP29], canine parvovirus 2 [CPaV2], *Pseudoalteromonas* phage PM2 [PM2], reovirus 3 [REO3], simian rotavirus A [SRVA]) had zinc, magnesium, and/or calcium binding sites listed in the UniProt database (95). SRVA, in particular, had several cation-binding sites that may contribute to the higher than predicted pI. These integral ions, in addition to polyvalent counterions retained in the core, might have a dramatic impact on the overall charge of some viruses. However, the degree to which these cations alter surface charge, as well as the irreversibility of many cation binding sites, remains to be determined.

Virions may also have a more nuanced permeability than models of soft or hard colloids. For example, some viruses (e.g., human rhinovirus, southern bean mosaic virus, and Mengo encephalomyocarditis virus) have selective cation channels located at capsid vertices (96, 97). Bacteriophage MS2 also has pores at its 5-fold axes that are ringed by disordered loops with a single glutamic acid at the apex (21). The negative charge of these loops above pH 4 may aid in selective diffusion of cations into the virion core and may help recruit and retain counterions to stabilize the negatively charged polynucleotide. Such a mechanism would further explain the lack of influence of the viral genome on virion charge.

All of the above factors might complicate a predictive model of virus pI. Whether a model can successfully incorporate or safely ignore these virion complexities

constitutes important future research. However, every confounding factor for a single model of virion charge lends support for an approach like PBR exclusion, which identifies functional virion structures rather than universally applying a simplified physical model. With expanded empirical *pI* data, more accurate *pI* prediction may be possible based on conserved virion structures. The PBR exclusion model has applications for researchers in water and wastewater treatment, as well as virus transport and microbial source tracking. As a general heuristic, viruses relying on electrostatic interactions between the polynucleotide and capsid proteins are more likely to have acidic *pIs* outside the circumneutral range expected from the sum of ionizable capsid residues. Thus, researchers may make use of the insights of the PBR exclusion method, even without identifying known PBRs or using the PBR prediction method developed by Heffron and Mayer (25). Future research should incorporate PBR exclusion into a quantitative model for virus surface charge. In addition, the PBR exclusion method gives rise to a conceptual electrostatic model of the virion that better unifies empirical evidence of virion structure and morphogenesis. This conceptual model is not an *ab ovo* assumption to account for a small subset of aberrant viruses. Instead, the PBR model follows from the success of the PBR exclusion method in accounting for both empirical *pIs* that align with capsid residue composition and empirical *pIs* that vary significantly. Further confirmation and refinement of this electrostatic model, particularly regarding the ionic composition of the virion core, could have far-reaching importance for structural virology in general.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

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