Research paper

Montmorillonite-mediated aggregation induces deformation of influenza virus particles

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A R T I C L E   I N F O

Article history:
Received 16 October 2015
Received in revised form 9 February 2016
Accepted 10 February 2016
Available online xxxx

Keywords:
Influenza A
Montmorillonite
Viruses
Aggregation

A B S T R A C T

The interaction between influenza virus [subtype A/Puerto Rico/8/1934 H1N1, (PR8)] and montmorillonite (Mt) is investigated by transmission electron microscopy and biochemical methods to determine how PR8 morphology and viability is affected. The majority of the PR8 particles formed aggregates with the Mt. TEM analysis showed that the virus particles retained structural integrity after aggregation but exhibited changes in morphology when compared to isolated PR8 and Mt aggregated with bromelain-treated PR8 (surface glycoproteins removed). Virus deformation shows that the virions exhibit an attraction to the Mt faces, possibly through hydrophobic interaction. The mean projection area of the aggregated PR8 was $(10.4 \pm 6.1) \times 10^3$ nm$^2$ compared to $(9.5 \pm 3.3) \times 10^3$ nm$^2$ for PR8 missing the surface glycoproteins; and $(8.0 \pm 3.9) \times 10^3$ nm$^2$ for non-aggregated PR8 controls. The increase in projection area of the aggregated PR8 suggests that the viruses deformed to increase contact region with the Mt faces with a subsequent compression normal to the face. PR8 missing the surface protein also exhibited an increase in projection area, although to a lesser extent, indicating that both the surface glycoproteins and viral envelope are attracted to the Mt faces. Circularity calculations indicate that the aggregated PR8 (circularity: 0.69 ± 0.16) are less round, i.e. more distorted, than either control PR8 (0.78 ± 0.14) or aggregated PR8 without surface glycoproteins (0.76 ± 0.12). The pleomorphic nature of influenza virus may allow it to survive the deformation induced by the Mt platelets. High resolution TEM micrographs revealed that the otherwise-round viruses flattened when in contact with platelet faces, thus increasing contact area with the Mt. PR8 was found to remain infectious after aggregation although at a lower rate than PR8 controls. The apparent reduced infectivity is likely a result of each aggregate (containing $10^2$ viral particles) acting as a single infectious unit.

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1. Introduction

Influenza is a virus whose interaction with clay minerals has not been investigated. It is a respiratory disease in humans; however, in waterfowl it is an infection of the gastrointestinal tract in which bird-to-bird transmission is fecal-oral. Infected birds secrete large numbers of virus particles into sediment-laden rivers and lakes (Dalton et al., 2009; Franklin et al., 2011; Ito et al., 1995; Nazir et al., 2011; Webster and Hulse-Poste, 2006; Webster et al., 1978). The residence time of

secreted influenza viruses in aquatic environments can vary from days to months and is influenced by suspended sediment and water chemistry, thereby affecting bird-to-bird transmission rates. A number of studies (Horn et al., 2011; Horn et al., 2012a; Horn et al., 2012b; Ito et al., 1995; Keeler et al., 2013; Vong et al., 2008) have found that influenza virus persists in lake and rainwater, soil, and waterfowl fecal matter for several months. In fact, avian influenza virus has been detected in water and sediment across the Atlantic seaboard of the United States after birds have departed indicating that environmental factors may extend virus stability. Therefore, it is of significant importance to determine how clay minerals affect the persistence of influenza virus in aquatic environments (Brown et al., 2009; Brown et al., 2007; Farnsworth et al., 2012; Negovetich and Webster, 2010; Stallknecht et al., 1990a; Stallknecht et al., 1990b).

The interaction between clay minerals and viruses can result in loss of infectivity, though this is not always the case (see Jin and Flury, 2002;
Kimura et al., 2008; Theng, 2012). In near neutral pH conditions such as encountered in natural waters virus adsorption by montmorillonite (Mt) is more effective than by illite or kaolinite (Theng, 2012). Viruses such as poliovirus are found to interact with non-aggregated Mt by adsorption to positively charged edges, enhancing virus survival (Vilker et al., 1983). A similar effect on viability was observed by Shirobokov (1968) for coxsackie virus and by Lipson and Stotzky (1983; 1986) for reovirus. Furthermore, mixtures of kaolinite and reovirus have been found to be more infectious and transported more readily than virus alone (Lipson and Stotzky, 1985).

Most studies of clay mineral-virus interaction have focused on non-enveloped viruses. For the enveloped bacteriophage, φ6, aggregation with Mt results in disassembly, rendering the virus inactive (Block et al., 2014). Influenza is a pleomorphic, enveloped virus commonly appearing with either a quasi-ellipsoidal or filamentous morphology. The viral envelope is covered with a high density of glycoprotein surface spikes (~450 spikes on a typical 130 nm diameter spherical virion (Booy et al., 1985; Harris et al., 2006; Katz et al., 2014)). Approximately 80% of the surface spikes are hemagglutinin (HA) and 20% are neuraminidase (NA) (Compans et al., 1970; Nayak et al., 2009). The protein spikes protrude approximately 14 nm radially from the envelope (Booy et al., 1985; Harris et al., 2006; Katz et al., 2014). There is evidence that the pleomorphic properties of influenza virus serve to protect it from puncture and mechanical stress such as might be encountered in environmental conditions (Li et al., 2011; Schaap et al., 2012; Serebryakova et al., 2011).

Based on the literature regarding the ability for non-enveloped and enveloped viruses to aggregate with clays, we predict that influenza virus particles will aggregate with clay minerals in the water column (Chrysikopoulos and Syngoua, 2012; Syngoua and Chrysikopoulos, 2012; Chrysikopoulos and Syngoua, 2013; Block et al., 2014; Syngoua and Chrysikopoulos, 2015). In this work, transmission electron microscopy (TEM) and biochemical analysis are employed to examine heteroaggregation of Mt and influenza A (subtype virus A/ Puerto Rico/8/1934 H1N1 (PR8) to (1) determine the degree to which montmorillonite interacts with the influenza virus leading to sequestration of the virus in the heteroaggregates; (2) the effect of aggregation on virus morphology; and (3) whether aggregated virus remains viable and capable of host infection.

2. Materials and methods

2.1. Montmorillonite

The clay mineral sample was a high-purity Na-montmorillonite [commercial name: “Accofloc”; chemical formula: (Na₄,Ca₃)₈.₃₃(Al₁.₆₇,Mg₀.₃₃)₄Si₄O₁₀(OH)₂·nH₂O; from American Colloid Company, Arlington Heights, IL]. Accofloc is a Na-Mt Volclay purified from Wyoming bentonite. Accofloc has a cation exchange capacity (CEC) of 79 meq/100 g (Sterte and Shabtai, 1987). Larger particles and non-clay minerals were removed by centrifugation at 3600 rpm (2700g) for 20 min. A 5% sodium hypochlorite (bleach) wash was used to remove organic contaminants from the Mt. This was followed by multiple washes in distilled water to remove any bleach residue. The supernatant comprising the colloidal fraction with an equivalent spherical Stokes diameter less than 0.2 μm was collected for the aggregation experiments and re-dispersed in distilled water. Purity of the Mt was confirmed by X-ray diffraction. By drying and weighing the stock dispersion, the Mt concentration (w/v) was determined to be 11 mg ml⁻¹. The equivalent Stokes diameter particle of a disk-like particles is given by: $d_s = \frac{3m}{4\pi\eta\phi}$, where $m$ is the particle mass and $\eta$ is the particle thickness (Jennings and Parslow, 1988). By considering Mt primary particles to be disk-shaped primary particles with a diameter of 0.5 μm as estimated from TEM micrographs, the average thickness of the 0.5 μm fraction is −35 nm for a primary particle volume of 6.9 x 10⁶ nm³ and based on an average density of 2.35 g cm⁻³, the average primary particle mass is 16 fg. Therefore a Mt concentration of 11 mg ml⁻¹ corresponds to −7 x 10¹¹ Mt primary particles ml⁻¹. The Mt dispersion was autoclaved immediately prior to the addition of influenza to ensure sterility.

2.2. Influenza A/Puerto Rico/8/1934 H1N1 virus (PR8)

PR8 is a low pathogenicity type A influenza virus isolated by Francis (1934) and is a representative influenza type A strain (Kelbourne and Murphy, 1960) used extensively since the 1960s. PR8 provides the genes needed for high growth in ovo by reassortment for influenza type A vaccine candidates and is also commonly used in laboratory experiments. PR8 is isomorphic to other influenza virus subtypes and is therefore a suitable model for the study of interactions between sediments and avian influenza. It has an isoelectric point of pH 5.3 (Michen and Graule, 2010). The majority of PR8 viruses in these experiments are roughly peanut-shaped with a 100 nm diameter envelope (Fig. 1). PR8 was replicated in embryonated chicken eggs (Charles River Laboratories International, Inc., Wilmington, MA) at the Geobiology Laboratory of the City College of New York. In accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of The City College of New York, protocol approval is not required for the use embryonated chicken eggs. All protocols were approved by the Biosafety Review Committee of The City College of New York.

Bromelain treatment was employed to cleave the HA and NA surface proteins from a subset of the PR8 samples (PR8₁₅₀₆₆) using the technique of Compans et al. (1970). Complete removal of surface glycoprotein spikes was confirmed by TEM analysis. The comparison of PR8 and PR8₁₅₀₆₆ Mt aggregates was used to elucidate the nature of the interaction of the glycoprotein spikes and viral envelope with the Mt.

2.3. Influenza-montmorillonite aggregates

PR8 in phosphate buffered saline, [PBS: P–3813 (138 mM NaCl, 2.7 mM KCl, 8 mM KH₂PO₄, 2 mM Na₂HPO₄) Sigma Aldrich, St. Louis, MO, USA] (pH 7.4, ionic strength 163 mM), was mixed with disaggregated Mt stock solution to obtain concentrations of 7 x 10¹⁰ plaque forming units (PFU) ml⁻¹ at a 10:1 ratio of Mt primary particles to virus particles. The density of the 56 kDa nucleoprotein bands by SDS-PAGE was used to prepare PR8₁₅₀₆₆ aggregates at similar concentration to the PR8 aggregated with Mt (PR8-Mt). The dispersions were allowed to aggregate at room temperature for 1 h and subsequently centrifuged at 2000 rpm (400g) for 5 min to pellet the aggregates (>0.5 μm equivalent Stokes diameter) and separate from the non-aggregated virus and Mt.
Mt particles that remained in dispersion. The pellets were washed three times with PBS. PR8-Mt, aggregates of PR8_{aggr} with Mt (PR8_{aggr-Mt}), and non-aggregated PR8 controls were applied to carbon-coated grids per standard TEM protocols and stained with phosphotungstic acid to enhance contrast for TEM analysis.

2.4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The fraction of PR8 in the aggregates was estimated by comparing the density of PR8 protein bands in coomassie-stained SDS-PAGE gels of the aggregated pellet and supernatant. A 4% acrylamide gel was stacked on top of a 12% separating gel. Electrophoresis was at room temperature at 20 mA for 1 h. The gels were stained with Colloidal Coomassie blue G 250 overnight.

2.5. Transmission electron microscopy

Micrographs for morphological analysis were acquired with a Zeiss 902 TEM operating at 80 keV with a 2048 × 2048 CCD detector. PR8 projection areas were determined for viruses in micrographs acquired at a magnification of 85,000× (0.512 nm/pixel) providing a 1.05 μm × 1.05 μm field of view. A total of 563 micrographs were analyzed, containing 4028 discrete virus particles: 2205 PR8 in PR8-Mt aggregates; 715 PR8_{aggr} in PR8_{aggr-Mt} aggregates; and 1108 non-aggregated PR8 controls. Morphology analysis was performed on the complete set of micrographs. In performing this analysis, care was taken to prevent duplicate counting of virus particles which appeared in the field of view of other micrographs. A JEOL 2100 TEM operating at 200 keV with a 2048 × 2048 CCD detector and at a magnification of 80,000× (0.13 nm/pixel) was employed to visualize details of PR8-Mt and PR8_{aggr-Mt} aggregates at higher magnification than with the 80 keV Zeiss TEM.

2.6. Image processing – projection area and circularity

In each micrograph, individual virus envelopes were manually traced using CorelDraw Version X4 (Corel Corp. Ottawa, Ontario, Canada). The glycoprotein spikes facilitated delineating the border of the viral envelopes; however, spikes were excluded from the traced regions and were not included in the calculation of projection areas. Only viruses for which the entire envelope could be clearly visualized were traced. In the case of overlapping viruses, if the entire envelope of a virus could be identified, its outline was traced and included in the morphology analysis; otherwise, the virus was excluded. Partially obscured viruses and those viruses for which the envelope could not be traced were excluded from the analysis.

The projection area was determined by counting the number of image pixels within each traced virus. All micrographs used in the projection area analysis were acquired at equal magnification on the Zeiss microscope.

A common measure of roundness of an object in an image is circularity (Cox, 1927). Circularity, C, is defined by C = 4πA / P^2, in which A is the area of the particle, P is the perimeter length, and 4π is a normalization factor to make the circularity of a perfect circle equal to 1. For all planar shapes, 0 < C ≤ 1. Virusretractperimeterswere calculated, using a chain code algorithm (Barba et al., 1992; Burger and Burge, 2009), on all particles for which the projection area was calculated. Perimeter calculations included the viral envelope but did not include the glycoprotein spikes. Digitization has the effect of increasing an object's perimeter and therefore decreasing circularity, however digitization will affect all perimeter calculations equally regardless of sample (PR8, PR8-Mt or PR8_{aggr-Mt}).

The circularity calculation software was tested using images containing a range of different diameter circles (equivalent to 40–200 nm for a 0.512 nm pixel size, data not shown) and yielded equal circularity, demonstrating that the circularity calculations are independent of object size over the range of sizes of PR8 in the micrographs. Using this algorithm, the maximum circularity that can be achieved for the digitized images is 0.92.

2.7. Infectivity titer

Measuring the infectivity of viruses in the Mt aggregates is complicated by the fact that each individual aggregate may act as a single infectious unit. In order to determine the diminishment of infectious units due to aggregation effects, PFU were quantified and compared to free virus. Three unique methods of assay were utilized to determine the viability of PR8 in the PR8-Mt aggregates – MDCK (Madin-Darby canine kidney) PFU titers, hemagglutination and egg-infectious dosage.

MDCK epithelial cells (ATCC® CRL-2936™) were grown in MEM (Minimum Essential Eagle Media containing Earle's salts and l-glutamine, supplemented with 5% of Fetal Bovine Serum, heat inactivated, Mediatech Inc., Manassas, VA) and in presence of penicillin and streptomycin (100 × solution, CELLGRO®, Mediatech Inc., Manassas, VA). Two milliliters of MDCK cells at a concentration of 2.25 × 10⁶ cells/ml were seeded into each well of a 6-well plate. After 24 h, the media was removed and the cells washed 3 times with ice-cold PBS. After which, 400 μl of 10^4, 10^5, 10^6-fold dilutions of virus samples in PBS were added to each well and incubated for 1 h at 37 °C with 5% CO₂. One well served as a negative control (no virus was added). The virus sample inoculum was aspirated and cells washed once with PBS. The 1% Seaplaque™ agarose media (2 × MEM/BSA media: 2 × MEM, 0.6% BSA fraction V, 2 × penicillin and streptomycin, 0.01% of DEAE-dextran and 0.75% of NaHCO₃ mixed 1 to 1 ratio with 2% Seaplaque™ agarose, Lonza, USA) was added to the cells. Cells with agarose overlay medium were incubated at 37 °C for 72 h, after which the agarose plug was discarded. Plates and cells were fixed with 10% formaldehyde solution in PBS, and then stained with crystal violet.

The hemagglutination assay was performed with 0.5% chicken red blood cells using the procedure of Hirst (1942). The number of PFU was determined using the 6.7 × 10^5 correlation coefficient of Gaussh and Smith (1968). Gaussh and Smith demonstrated that egg infectious dose by HA assay correlates with PFU determined by culture of MDCK cells. PR8 was mixed with PBS and disaggregated Mt stock to obtain approximate concentrations of 3 mg ml⁻¹ of Mt and 1.7 × 10^⁵ PFU/ml of influenza particles. After 1 h incubation, the dispersion was centrifuged at 2000 rpm (400g) for 5 min to separate aggregates from non-aggregated virus and Mt platelets, and the supernatant was removed. The pellet was washed 3 times with PBS followed by centrifugation at 400g for 5 min, and re-dispersed in PBS to maintain the sample volume. The supernatant was collected after the first spin. Samples of both the final pellet and the initial supernatant were collected for SDS-PAGE analysis, and end point dilution following the procedure of Reed and Muench (1938). Egg infectious dosage was conducted by serial dilution of both the pellet and the supernatant using PBS containing penicillin and streptomycin. 10-day-old embryonated chicken eggs were inoculated with 100-fold dilutions and duplicated to confirm results. After 72 h of incubation at 37 °C, the allantoic liquid was collected, and analyzed by hemagglutination assay. Two-fold dilutions of each sample were mixed with 0.5% chicken red blood cells (Innovation Research, Inc., Novi, MI) in PBS followed by incubation at room temperature for 30 min.

3. Results and discussion

3.1. Formation of aggregates of influenza PR8 and Mt

Mt dispersed with PR8 and PR8_{aggr} influenza formed aggregates within 30 min of mixing. Low speed centrifugation separated non-aggregated Mt and individual virions from PR8-Mt aggregates > 0.5 μm in size. SDS-PAGE (Fig. 2) of the non-aggregated PR8 and Mt supernatant and aggregated pellet fractions reveals that most of the PR8
aggregated with the Mt while only trace amounts of PR8 remained non-aggregated in the supernatant.

3.2. TEM of PR8 aggregated with Mt

Micrographs of aggregates of PR8-Mt and PR8$_{δHN}$-Mt are shown in Fig. 3a and b, respectively. In both micrographs several viral particles are adjacent to platelets. Although TEM preparation results in the majority of Mt platelets to be oriented in the x,y-plane (i.e., the detector plane which is orthogonal to the electron beam), the flattening of virions against the platelet faces is more discernible when platelets are oriented orthogonal to the x,y-plane. Higher magnification views of PR8 particles in PR8-Mt aggregates are shown in Fig. 4a, b, and c. The virus particles in contact with platelets exhibit varying degrees of deformation. An aggregated PR8 in which the glycoprotein spikes have been removed by bromelain treatment (PR8$_{δHN}$) is presented in the micrograph of Fig. 4d. The virus observed in Fig. 4a is round, but exhibits flattening of viral envelope where the virus is in contact with the platelet face. The glycoprotein spikes in contact with Mt are discernible but appear shorter than spikes that are not in contact with Mt. The upper part of the virus observed in Fig. 4b is in contact with an Mt platelet and exhibits significant flattening of the viral envelope. In this region, the glycoprotein spikes appear distorted and noticeably shorter, indicating that HA and NA spikes have undergone a conformational alteration. The length of glycoprotein spikes not in contact with Mt platelet is consistent with values reported in the literature (13–14 nm). The virion in Fig. 4c is sandwiched between two platelets resulting in significant deformation of the virus particle and an increase in contact area with the platelets. The glycoprotein spikes are discernible only in areas not in contact with Mt (Fig. 4c). PR8$_{δHN}$ in Fig. 4d is attached to a platelet face indicating that deformation can occur in the absence of glycoprotein spikes. A non-deformed PR8 control exhibiting intact spikes surrounding the envelope is shown in Fig. 5.

3.3. Morphological analysis of PR8 in aggregates

TEM micrographs were analyzed to determine morphological changes to PR8 and PR8$_{δHN}$ after aggregation. The mean values for the virus projection area and a related parameter, circularity, are discussed below and summarized in Table 1.

Projection area size distributions for PR8 and PR8$_{δHN}$ aggregated with Mt, and PR8 controls are shown in Fig. 6a, b and c. Corresponding box plots with 5% and 95% limits are shown in Fig. 6d. The projection area of PR8 on average is greater than PR8$_{δHN}$ in Mt aggregates, and both have projection areas greater than PR8 controls. The interquartile range (IQR) of projection area values of PR8 in the PR8-Mt is 6414 nm$^2$, 70% broader than the IQR of PR8$_{δHN}$ (3734 nm$^2$) and 60% broader than the IQR of PR8 controls (3916 nm$^2$), here attributed to particle distortion due to the affinity of glycoproteins to the Mt face. A review of the literature reveals no mechanism for increasing the total volume of the virions as a result of aggregation with Mt, therefore, the observed projection area increase is a result of virus expansion in the x,y-plane (i.e. plane of the micrographs which is orthogonal to the microscope axis) with a corresponding compression along the z-axis to conserve overall volume. The relationship between increase projection area as a result of a change in morphology is shown schematically in Fig. 7. The tendency for Mt platelets to adopt a preferred flat orientation in the x,y-plane, indicates that the virus deformation is also an expansion in the plane of the Mt platelet face. This expansion increases contact area between the virus and the platelet. The increased projection area of
PR8 and PR8δHN in Mt aggregates implies an affinity between the virus particles and platelet faces. However, the larger mean projection area of PR8 signals a stronger attachment between glycoprotein spikes and negatively charged regions of Mt than between the spike-less envelope and Mt.

Circularity provides a measure of distortion, as distorted particles tend to have a greater perimeter. Low circularity may be the result of an irregular perimeter (e.g. starfish shaped) or extreme elongation (e.g. a high-eccentricity ellipse) (Kröner and Doménech Carbó, 2013). PR8 in the Mt aggregates exhibits a lower circularity than PR8δHN and PR8 controls. The mean circularity of PR8δHN is nearly equal to PR8 control indicating that the degree of deformation of PR8δHN is less than for PR8 in the aggregate. In Mt-aggregated PR8, the lower circularity is related to an increase in elongation, reinforcing projection area

**Table 1**

<table>
<thead>
<tr>
<th>Sample</th>
<th>N</th>
<th>Mean PR8 Projection area (nm²)</th>
<th>Mean PR8 Circularity</th>
</tr>
</thead>
<tbody>
<tr>
<td>PR8-Mt</td>
<td>2205</td>
<td>$(10.4 \pm 6.1)$ × 10³</td>
<td>0.69 ± 0.16</td>
</tr>
<tr>
<td>PR8δHN-Mt</td>
<td>715</td>
<td>$(9.5 \pm 3.3)$ × 10³</td>
<td>0.76 ± 0.12</td>
</tr>
<tr>
<td>PR8 control</td>
<td>1108</td>
<td>$(8.0 \pm 3.9)$ × 10³</td>
<td>0.78 ± 0.14</td>
</tr>
</tbody>
</table>

Please cite this article as: Block, K.A., et al., Montmorillonite-mediated aggregation induces deformation of influenza virus particles, Appl. Clay Sci. (2016), http://dx.doi.org/10.1016/j.clay.2016.02.010
observations of a strong affinity with Mt platelet faces. The frequency distributions of circularity values for PR8 in the aggregate, PR8δHN, and PR8 controls are plotted in Fig. 8a, b, and c, respectively and with a corresponding box plot in Fig. 8d. The IQR of circularity values of the PR8 control is 0.129, similar to the IQR of 0.139 for PR8δHN, both of which are significantly different from the IQR of 0.243 for PR8 in the aggregate. This strongly suggests that the presence of glycoprotein spikes results in a different deformation response to aggregation of influenza and Mt.

3.4. PR8 infectivity after aggregation with Mt

PR8 infectivity after Mt aggregation relative to non-aggregated virus was compared by determining the number of PFU/ml in MDCK cells. The aggregate pellets exhibited 10²-fold reduction in PFU count. Since the average aggregate size was ~2 μm and contained approximately 10² vi- rions, the reduction in PFU is likely a result of each aggregate acting as a single PFU rather than a reduction in individual virus infectivity. The infectivity of the supernatant was a factor of 10⁵ lower than the control indicating that almost no virus remained non-aggregated, consistent with the SDS-PAGE analysis. The PFU/ml count is summarized in Table 2.

As confirmation of the MDCK assay, egg infectious dosages of the pellet and supernatant were compared to PR8 controls. Although the initial PR8 concentration was higher than that used in the MDCK assay, this assay confirmed the 10²-fold reduction in PFU for the pellet (Table 2). The egg assay confirmed that less than 1% of the PR8 remained in the supernatant.

The infectivity of PR8 depends on the availability of hemagglutinin surface glycoproteins to bind to cells. The third assay was a hemagglutination assay which determines the availability of hemagglutinin in the aggregates. The assay indicated 20× less available hemagglutinin in PR8-Mt than in PR8 controls (Table 2). That the hemagglutination assay exhibited only a 20-fold reduction instead of 10² likely resulted from several PR8 situated near the aggregate surface, contributing to the hemagglutination assay, but acting as a single infectious unit in the MDCK and egg assays.

3.5. Implications for enveloped viruses in the environment

The PR8 viability results have profound implications for the transmission of influenza, specifically avian influenza in aquatic systems. The PR8-Mt complex effectively behaves as an infectious unit, with all virus particles within the aggregate potentially infecting a host. Influenza occurs in multiple subtypes, therefore, in natural waters the ingestion of virus-sediment aggregates by birds could serve as a natural pathway for co-infection in the bird gut, possibly leading to new virulent strains. Furthermore, while previous studies have found that natural waters shorten the residence time of influenza compared to buffered distilled water and filtered natural water, the results suggest that the sediment beds may constitute a significant reservoir for influenza.

The pleomorphism of influenza imparts pliability to the particle, allowing the virus to remain intact despite the significant deformation produced by interaction with Mt. This is in contrast to icosahedral enveloped viruses such as the bacteriophage, φ6 (a member of the Cystoviridae family) which is inactivated by partial disassembly of the viral envelope after aggregation with Mt (Block et al., 2014). There is evidence that the abundant glycoprotein spikes on PR8 may in fact contribute to the robustness of influenza in the environment. Using atomic force microscopy, Schaap et al. (2012) found that wild-type influenza experienced 30% deformation occurring in two phases as a result of envelope stiffness. In the absence of surface spikes the envelope stiffness increased, requiring less force and resulting in only one deformation phase. While the HA glycoprotein spikes exhibit flexibility in the neck
region (Serebryakova et al., 2011), they also add mechanical stability to the influenza virus (Schaap et al., 2012). Lysosomes made from influenza lipid envelopes have been shown to be highly flexible and deform up to 20% (Li et al., 2011). Morphological analysis, specifically the relative increases in projection area of PR8 and PR8\textsubscript{aggreg} as a result of aggregation, is consistent with these findings. More generally, this strongly suggests that for environmental viruses the effect of aggregation with sediments on viability and residence time depends largely on viral structure.

3.6. Conclusions

PR8 readily aggregates with Mt in dispersion. The changes in morphology of the virus in response to contact with Mt confirm that the influenza virus envelope readily deforms without compromising structural integrity. The pleomorphic structure of PR8 allows it to withstand stresses during interaction with charged environmental components such as colloidal clay mineral sediments while remaining infectious. Both PR8 and PR8\textsubscript{aggreg} predominantly exhibit attraction to the negatively charged platelet faces. HA spikes are negatively charged and the experiments were conducted at pH 7, above the isoelectric point of PR8, suggesting that the interaction is not electrostatic but may instead be hydrophobic or van der Waals.

Given the findings in this study that each PR8-Mt aggregate contains $\sim 10^2$ virus particles, it is here speculated that in aquatic environments, aggregation with dispersed sediment may increase the likelihood for multiple influenza virus subtypes to be contained in close proximity, i.e. within an aggregate, possibly facilitating natural co-infection after ingestion by waterfowl.

Acknowledgments

This work was supported in part by City Seed Grant #93370-09 from The City College of New York; PSC-CUNY Grant #67709-00 45 from the City University of New York; the Research Centers in Minority Institutions (NIH/NCRR/RCMI) CCNY/Grant G12-RRO3060; and a National Institute of General Medical Science Grant SC1-GM092781.

References


Table 2

<table>
<thead>
<tr>
<th>PR8 sample</th>
<th>PR8 infectivity (PFU/ml)</th>
<th>Hemagglutination</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDCK</td>
<td>Egg titer*</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>$5.7 \times 10^4$</td>
<td>$10^6$</td>
</tr>
<tr>
<td>Pellet</td>
<td>$6.5 \times 10^6$</td>
<td>$10^8$</td>
</tr>
<tr>
<td>Supernatant</td>
<td>$&lt; 10^2$</td>
<td>$1.3 \times 10^5$</td>
</tr>
</tbody>
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* This value represents the highest sample dilution resulting in infection of an egg.

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