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Research paper

Montmorillonite-mediated aggregation induces deformation of influenza virus particles

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ABSTRACT

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. The 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.

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 (Horm et al., 2011; Horm et al., 2012a; Horm 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 long 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;

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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 Syngouna, 2012; Syngouna and Chrysikopoulos, 2012; Syngouna and Chrysikopoulos, 2013; Block et al., 2014; Syngouna 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: $(Na,Ca)_{0.33}(Al_{1.67}Mg_{0.33})Si_4O_{10}(OH)_2 \cdot nH_2O$; 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 redispersed 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 $d_s \approx 1.5(\delta t)^{½}$ were δ is the particle diameter and t 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 0.2 μm fraction is ~35 nm for a primary particle volume of 6.9×10^6 nm^3 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 ~7×10^{11} 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 (Kilbourne 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 ($PR8_{\delta HN}$) using the technique of Compans et al. (1970). Complete removal of surface glycoprotein spikes was confirmed by TEM analysis. The comparison of PR8 and $PR8_{\delta HN}$ in 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 KH_2PO_4, 2 mM Na_2HPO_4) Sigma Aldrich, St. Louis, MO, USA] (pH 7.4, ionic strength 163 mM), was mixed with disaggregated Mt stock solution to obtain concentrations of 7×10^{10} 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 $PR8_{\delta HN}$ 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

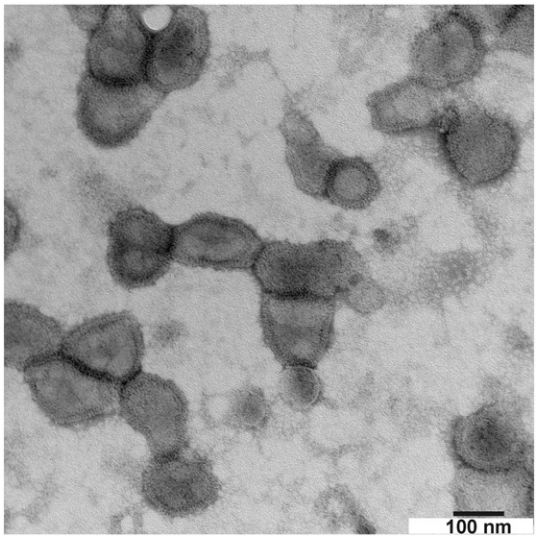

Fig. 1. Electron micrograph of peanut-shaped PR8 particles.