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Full inactivation of alphaviruses in single particle and crystallized forms

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A B S T R A C T

Inherent in the study of viruses is the risk of pathogenic exposure, which necessitates appropriate levels of biosafety containment. Unfortunately, this also limits the availability of useful research instruments that are located at facilities not equipped to handle infectious pathogens. Abrogation of viral infectivity can be accomplished without severely disrupting the physical structure of the virus particle. Virus samples that are verifiably intact but not infectious may be enabled for study at research facilities where they would otherwise not be allowed. Inactivated viruses are also used in the development of vaccines, where immunogenicity is sought in the absence of active infection. We demonstrate the inactivation of Sindbis alphavirus particles in solution, as well as in crystallized form. Inactivation was accomplished by two different approaches: crosslinking of proteins by glutaraldehyde treatment, and crosslinking of nucleic acids by UV irradiation. Biophysical characterization methods, including dynamic light scattering and transmission electron microscopy, were used to demonstrate that the glutaraldehyde and UV inactivated Sindbis virus particles remain intact structurally. SDS-PAGE was also used to show evidence of the protein crosslinking that was expected with glutaraldehyde treatment, but also observed with UV irradiation.

1. Introduction

It is possible to modify the chemistry of a virus in a manner that abolishes infectivity but does not significantly alter the physical structure. Such modification enables biophysical studies to be carried out on viruses without the pathogenic risks that would otherwise prevent the use of instrumentation and techniques that are not compatible with the appropriate biosafety regulations. We report here two different approaches to thus inactivate the Sindbis virus, both in single particle and crystallized form.

Sindbis virus (SINV) is one of 32 viruses currently classified in the family *Togaviridae*, which also includes Chikungunya virus (CHIKV), Semliki Forest virus (SFV), Ross River virus (RRV), and Rubella virus (RUBV) (Adams et al., 2015, 2014). SINV, CHIKV, SFV and RRV are arboviruses that are carried by mosquitoes and transmitted to humans or other vertebrates. *Togaviridae* are evolutionarily linked to *Flaviviridae* (Strauss and Strauss, 2001), which includes other structurally similar arboviruses such as Yellow Fever virus (YFV), West Nile virus (WNV), dengue virus (DENV), and Zika virus (ZIKV). Sindbis virus is characterized by a host-derived lipid membrane envelope, a positive-strand RNA genome, and three structural proteins (C, E1, E2) of which there are 240 copies each. The RNA genome is enclosed within an assembly of the capsid (C) proteins. This capsid is surrounded by the lipid envelope which anchors the E1 and E2 envelope proteins. E1 and E2 associate to form heterodimers, which further assemble into 80 trimeric 'spikes' that protrude to the exterior from the lipid envelope. The capsid proteins and the envelope proteins are both arranged icosahedrally with a T = 4 symmetry (Paredes et al., 2005, 1993). Small angle neutron scattering measurements determined the precise diameter of Sindbis virus as 67.6 ± 2.5 nm at pH 7.2, and 72.0 ± 2.8 nm at pH 6.4 (He et al., 2012). The Sindbis virus is known to cause rashes, fever, joint and muscle pain, and symptoms can be chronic in some cases (Adouchief et al., 2016). It can be routinely propagated to very high densities under biosafety level 2 conditions (Hernandez and Brown, 2005), and it is used as a prototypical model of study for the alphavirus genus of *Togaviridae* (Adams et al., 2014).

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A variety of direct treatment methods have been described that will effectively abolish the infectivity of a virus for the purposes of sanitation, but not always with an interest in preserving the physical structure (Mcdonnell and Russell, 1999). Inactivation methods that are not reliant on genetic manipulation or irreversible physical denaturation include chemical crosslinking (aldehydes, β-propiolactone), radiation exposure (UV, gamma), and alkylation of nucleic acids (β-propiolactone, binary ethylene imine). These methods have been used to partially or fully inactivate a variety of enveloped and non-enveloped viruses (Delrue et al., 2012).

Of the aldehydes, glutaraldehyde ($C_5H_8O_2$) is perhaps the most reactive and efficient at producing stable protein crosslinks (Bowes and Cater, 1968; Nimni et al., 1987). The various mechanisms by which glutaraldehyde crosslinking may occur are dependent on the multiple molecular conformations enabled by its five carbon dialdehyde nature (Migneault et al., 2004). Although the mechanisms are not entirely predictable and have not been fully described, the reaction usually begins with nucleophilic addition from the ε-amine group of lysines, and can proceed to crosslink within a protein chain or between separate, but adjacent protein chains. Glutaraldehyde crosslinking is only slightly reversible at physiological pH (pH 7–pH 9) (Okuda et al., 1991). Other nucleophilic functional groups in proteins have also been shown to be reactive with glutaraldehyde, including thiol (cysteine), phenol (tyrosine), imidazole (histidine), and the free α-amino group on the N-terminal residue (Avrameas and Ternynck, 1969; Habeeb and Hiramoto, 1968). Formaldehyde has also been shown to be reactive with these functional groups in forming crosslinks, in addition to amide (asparagine, glutamine), guanidyl (arginine), and indole (tryptophan) groups (Fraenkel-Conrat and Mecham, 1949; Fraenkel-Conrat and Olcott, 1948; French and Edsall, 1945). Formaldehyde is known to also react with nucleic acids to form crosslinks (Huang and Hopkins, 1993; Lu et al., 2010), but this activity is restricted with glutaraldehyde (Hopwood, 1975; Sewell et al., 1984).

UV radiation is also capable of inactivation by various mechanisms (Ravanat et al., 2001). Nucleic acids absorb within the UVC (100–280 nm) and UVB (280–320 nm) range, with maximal absorbance near 260 nm. Direct UVC and UVB exposure induces nucleic acid crosslinking between adjacent pyrimidines, forming either *cis-syn* cyclobutane pyrimidine dimers (CPDs) or pyrimidine [6–4] pyrimidone photoproducts ([6–4] PPs) as the major crosslinks. Both photoproducts are produced efficiently in DNA under UVC conditions (254 nm), and it appears that CPDs become relatively more prevalent as the wavelength increases into the UVB range (Ravanat et al., 2001; Yoon et al., 2000). UV radiation also catalyzes the formation of reactive oxygen species (from either endogenous or exogenous sensitizer molecules) that go on to indirectly cleave or otherwise damage nucleic acids (Ravanat et al., 2001) as well as proteins (Eischeid and Linden, 2011; Wigginton et al., 2012, 2010). Crosslinking reactions are also known to take place between proteins and nucleic acids, primarily involving thymine reacting with lysine (Saito and Matsuura, 1985; Shetlar et al., 1975), arginine, and cysteine (Schott and Shetlar, 1974). Protein to protein crosslinking has not been extensively studied, but has been observed as well (Prinsze et al., 1990). Although most investigation into the effect of UV on nucleic acids is targeted on DNA, the principles can generally be extended to RNA as well.

Although decades of research have been invested into describing the various effects of aldehydes or UV irradiation on nucleic acids and proteins in isolation, the understanding of these processes in the macromolecular context of a virus is still quite limited (Wigginton et al., 2012). The proximity of a condensed viral genome to capsid proteins, and the tight multimeric packing of these proteins produces a high potential for a variety of cross reactions to occur under the appropriate conditions.

2. Experimental methods

2.1. Preparation and purification of Sindbis virus

Methods for the cultivation, purification, and quantification of Sindbis virus were adapted from the established protocols by Hernandez and Brown (2005). Baby hamster kidney (BHK) cells were cultivated by passaging in minimal essential medium (MEM) with supplements (5% fetal bovine serum, 5% tryptose phosphate broth, 2 mM L-glutamine, 50 μg/mL gentamicin). Cells were grown in 875 cm^2 multi-level flasks until nearly confluent in preparation for large-scale infections. A multiplicity of infection (MOI) of 0.1 was used to inoculate, and the cells were maintained during the infection period in Glasgow minimal essential medium (GMEM) containing 2 g/L $NaHCO_3$ in addition to the other supplements used with MEM. Following a 24 h infection period, growth medium was clarified by centrifugation at 1000 × *g* for 10 min to remove cell debris.

The harvested virus was purified using isopycnic sedimentation. Continuous gradients of potassium tartrate (dibasic) in PN buffer (50 mM PIPES pH 7.2, 100 mM NaCl) were prepared in 19 mL volumes with a 15–37% concentration range. Gradients were overlayed with 20 mL of the clarified supernatant. Sindbis virus was sedimented in the continuous gradient by ultracentrifugation in rotor SW 32 Ti (Beckman Coulter) at 100,000 × *g* for 4 h at 4 °C, and virus bands were extracted by bottom puncture of the 40 mL ultracentrifuge tubes. Extracted virus bands were pooled, dialyzed in PN buffer to remove potassium tartrate, and applied to a second round of continuous gradient purification to concentrate the virus band into a smaller volume. Dialysis in PN buffer was repeated to remove potassium tartrate.

An additional sedimentation step was applied for preparation of the sample used in crystallization. Following dialysis, the purified virus in PN buffer was centrifuged for 2 h at 100,000 × *g* in rotor SW 55 Ti (Beckman Coulter) to pellet the virus, which was resuspended in PN buffer to a concentration of 1 mg/mL (~3×10^{11} pfu/mL). Concentration was determined by a modification of the Lowry Assay method (Markwell et al., 1978).

2.2. Crystallization

Methods for producing microcrystals of Sindbis have been previously reported (Lawrence et al., 2015), and are based on previously established methods for macrocrystal production (Harrison et al., 1992). Briefly, crystallization was induced by vapor diffusion using the hanging drop method. Hanging drops consisted of equal parts of 1 mg/mL Sindbis virus in PN buffer and the precipitant solution in a 10 μL volume. The precipitant was 5.5% w/v PEG 8000, 7.5% w/v glycerol, and 0.24 M KCl, and it was added to each reservoir in a 0.5 mL volume. Crystals began to appear in hanging drops after 5–6 days of equilibration at 4 °C. Microcrystals with a diameter of 10–20 μm were produced at a density of approximately 10^6 crystals/mL.

2.3. UV irradiation

Samples of purified Sindbis virus were exposed to increasing doses of UVC radiation to determine the minimum amount required for full inactivation. A Stratalinker UV Crosslinker Model 1800 was used to generate 254 nm UVC radiation at a rate of 60 μjoules/cm^2/s.

Single particle samples of Sindbis virus suspended in PN Buffer at a concentration of 0.07 mg/mL (~2×10^{10} pfu/mL) were subjected