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Monolithic anion-exchange chromatography yields rhinovirus of high purity

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ABSTRACT

For vaccine development, 3D-structure determination, direct fluorescent labelling, and numerous other studies, homogeneous virus preparations of high purity are essential. Working with human rhinoviruses (RVs), members of the picornavirus family and the main cause of generally mild respiratory infections, we noticed that our routine preparations appeared highly pure on analysis by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), exclusively showing the four viral capsid proteins (VPs). However, the preparations turned out to contain substantial amounts of contaminating material when analyzed by orthogonal analytical methods including capillary zone electrophoresis, nano electrospray gas-phase electrophoretic mobility molecular analysis (nES GEMMA), and negative stain transmission electron microscopy (TEM). Because these latter analyses are not routine to many laboratories, the above contaminations might remain unnoticed and skew experimental results. By using human rhinovirus serotype A2 (RV-A2) as example we report monolithic anion-exchange chromatography (AEX) as a last polishing step in the purification and demonstrate that it yields infective, highly pure, virus (RV-A2 in the respective fractions was confirmed by peptide mass fingerprinting) devoid of foreign material as judged by the above criteria.

1. Introduction

Picornaviruses are icosahedral particles of 30 nm diameter with a single-stranded (+) RNA genome enclosed within a non-enveloped protein shell composed of 60 copies each of the four capsid proteins VP1 through VP4. Physicochemical and structural analyses require sizable quantities of these viruses at high purity. Virus is usually recovered from infected tissue culture cells via lysis and purified by differential centrifugation followed by ultracentrifugation on sucrose, potassium tartrate, Nycodenz, or cesium chloride density gradients (e.g. references (Ashley and Caul, 1982; Gugerli, 1984; Liu et al., 2011)). Virus particles are recovered from selected fractions, pelleted, and resuspended in a suitable buffer. Working with RVs, which belong to the genus *Enteroviruses,* and cause more than 50% of all (mostly mild) respiratory infections, we noticed that routine preparations showed exclusively the four viral capsid proteins (VPs) upon sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). However, Kremser et al. (Kremser et al., 2006) already demonstrated, by using capillary electrophoresis with UV absorbance detection (CE-UV) that RV-A2 purified via ultracentrifugation on a sucrose density gradient contained some additional material ('contaminant'). This material showed up as peak monitored at 200 nm UV absorption (typical wavelength for RV-A2 analysis applied in CE separation) in CE runs in presence of SDS above its critical micellar concentration in the background electrolyte (Kremser et al., 2006). Nevertheless, due to the low absorbance of this contaminant in relation to the virus, it was considered negligible for most experiments. However, when labelling RV-A2 with amine-reactive NHS-ester dyes, the contaminant became strongly fluorescent as seen in CE with laser induced fluorescence detection (CE-LIF), suggesting either the presence of many primary amino groups or non-covalent attachment of the dye to hydrophobic surface patches or pockets of the contaminant (Kolivoska et al., 2007; Kremser et al., 2004; Weiss et al., 2007).

Analysis of these RV-A2 preparations by negative-stain transmission

Abbreviations: ACN, acetonitrile; AEX, anion-exchange chromatography; CE, capillary electrophoresis; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EM, electrophoretic mobility; FCS, fetal calf serum; GEMMA, gas-phase electrophoretic mobility molecular analyzer; RV, human rhinovirus; RV-A2, RV-B14, RV-A16, RV-A89, RV serotypes; LIF, laser induced fluorescence; MALDI, matrix-assisted laser desorption ionization; MEM, minimal essential medium; MS, mass spectrometry; MOI, multiplicity of infection; nES, nano electrospray; PAGE, polyacrylamide gel electrophoresis; PMF, peptide mass fingerprint; PSD, post-source decay; SDS, sodium dodecyl sulfate; SMEM, spinner minimum essential medium; $TCID_{50}$, tissue culture infectious dose 50; TEM, transmission electron microscopy; TFA, trifluoroacetic acid; VP, viral protein

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electron microscopy (TEM) revealed cloud- or 'popcorn'-shaped amorphous masses (Bilek et al., 2009). By the same token, analysis by means of a nano Electrospray Gas-phase Electrophoretic Mobility Molecular Analyzer (nES GEMMA) (Kaufman et al., 1996) revealed the presence of two similarly-sized components (Bacher et al., 2001); the virus gave a sharp, and the second component a very broad peak indicating polydisperse particles (Weiss et al., 2012). Both sample components shared similar apices at about 30 nm dry particle diameter.

nES GEMMA separates single-charged nanoobjects obtained from a nES process with subsequent charge reduction in a bipolar atmosphere induced by a ^{210}Po α-particle source or a soft X-ray beam in the gas-phase at ambient pressure. The analytes become separated according to their electrophoretic mobility (EM) diameter in a tunable electric field and an orthogonal constant laminar high-sheath flow of particle-free air. Only particles of a given EM diameter (corresponding to the dry particle size in case of spherical analytes) can pass the size analyzer at a given voltage. The latter is varied to scan a predetermined size range. Detection is based on scattering of a laser beam focused on the separated, monodisperse analytes that create droplets in the supersaturated *n*-butanol or water atmosphere of the detector unit. Thus, particle counting is independent of their chemical nature resulting in number-particle concentrations, as suggested by the European Commission for nanoparticle characterization (2011/696/EU from October 18th, 2011). nES GEMMA has proven useful for the analysis of intact virions by quite a number of laboratories (see e.g. (Bacher et al., 2001; Guha et al., 2012; Havlik et al., 2014; Hogan et al., 2006; Pease, 2012; Thomas et al., 2004; Wick, 2015; Wick and McCubbin, 1999)). In contrast to the CE-UV measurements, the results of nES GEMMA estimated the concentration of the contaminating material rather close to the concentration of RV-A2. This is in agreement with the ratio between viral particles and the amorphous masses observed in negative stain TEM (Weiss et al., 2012).

A fortuitously obtained sample strongly enriched in the contaminant allowed its characterization via CE-UV and nES GEMMA, as well as its confirmation as the popcorn-shaped masses in negative-stain TEM (Weiss et al., 2012). Based on the above observations we suspect that it is a heterogeneous, polydisperse mixture of membranous material, possibly derived from exosomes, since lipase treatment led to its partial disappearance (Weiss et al., 2015a). This latter lipase-treated sample allowed us to finally prepare highly pure empty capsids to determine their M_r via native electrospray ionization mass spectrometry (native ESI MS). The results were in excellent accordance with values calculated from the sum of the building blocks, i.e. 60 copies each of VP1, VP2 and VP3 – note that native virus contains, in addition, 60 copies of VP4 and the genomic RNA of about 7200 to 7300 bases in length (the variation is due to the heterogeneous length of the 3′-poly (A) tail; (Weiss et al., 2015a)). Nevertheless, the purity of our virus preparations was still variable despite lipase treatment (Weiss et al., 2016). This prompted us to assess the utility of anion exchange chromatography (AEX) on a monolithic column for virus purification. AEX was reported to aid for instance the purification of the rod-shaped tomato mosaic virus, hepatitis A virus, calicivirus, PRD1 phage, mumps and measles virus and another picornavirus (enterovirus 71) even leading to a considerable increase of virus concentration in the column eluates (Kovac et al., 2009; Kramberger et al., 2004; Oksanen et al., 2012; Sviben et al., 2017; Venkatachalam et al., 2014). In the following, we demonstrate that AEX also yields a pure RV-A2 fraction well separated from other non-viral components, including ferritin and proteasomes, and free of the 'contaminant' as shown by negative stain TEM and nES GEMMA. MALDI mass spectrometry was used to confirm the presence of RV-A2 in the main AEX fraction.

2. Materials and methods

2.1. Chemicals and reagents

Dithiothreitol (DTT, BioUltra), 2-mercaptoethanol (> 98%), iodoacetamide (BioUltra), trifluoroacetic acid (TFA, > 99%), potassium ferricyanide (III) (approx. 99%), tris(hydroxymethyl)aminomethane, sodium chloride, magnesium chloride hexahydrate (Bioreagent), ammonium acetate (NH_4OAc, 99.99% trace metal basis) and ammonium hydroxide (28.2% NH_3) for pH adjustment of NH_4OAc were purchased from Sigma Aldrich (Steinheim, Germany). Ammonium hydrogen carbonate (NH_4HCO_3, > 99.5%) was obtained from Fluka (Buchs, Switzerland). Acetonitrile (ACN, for analysis, *p.a.*) and formic acid (*p.a.*) were purchased from Merck (Darmstadt, Germany), sodium thiosulfate pentahydrate (*p.a.*) from Riedel-de Haen (Seelze, Germany), trypsin/LysC (MassSpec grade) from Promega (Madison, WI, USA), RNase and DNase from Roche (Mannheim, Germany), trypsin from Life Technologies (Carlsbad, CA, USA). Water used in this study was prepared by means of a Millipore (Billerica, MA, USA) apparatus (18.2 MΩ cm resistivity at 25 °C). Sucrose was obtained from Carl Roth (Karlsruhe, Germany). L-glutamine (2 mM in total from Gibco, Grand Island, NY, USA), Pen-Strep (100 U/mL penicillin, 100 µg/mL streptomycin in total from Lonza, Verviers, Belgium) and heat inactivated FCS (10% in total from Sigma Aldrich) were supplemented to the cell growth medium.

2.2. RV-A2 preparation

RV-A2 was purified following the protocol detailed in 2015 (Weiss et al., 2015b). In brief, HeLa-H1 cells grown in four 3 L spinner flasks to about 1×10^6 cells/mL were resuspended in fresh SMEM (Sigma Aldrich), adjusted to 2 mM $MgCl_2$ and supplemented with 2% horse serum (Gibco), infected at MOI = 1, and stirred with 15 rpm at 34 °C for 16.5 h. The low MOI and long incubation time results in two consecutive infection cycles with the first one producing sufficient progeny virus for all cells to become infected in the second round. The conditions were chosen such that the majority of the cells are not yet lysed. They were thus broken with a tight-fitting Dounce homogenizer to set free the intracellular virus. Cell debris was removed by low speed centrifugation and virus in the supernatant was pelleted at 8.0×10^4 *g* for 2 h in a TI45 Beckman rotor (Brea, CA, USA) at 4 °C. The pellets were resuspended in 4 mL 20 mM Tris-HCl (pH 7.5), 10 mM EDTA (Merck), and contaminating nucleic acids as well as proteins were digested with DNase and RNase-A (10 min each at ambient temperature) followed by trypsin (5 min at 37 °C). N-laurylsarcosine (Sigma Aldrich) was added for overnight incubation at 4 °C. Aggregated material was removed in an Eppendorf benchtop centrifuge at full speed for 15 min and the supernatant was layered on top of four sucrose density gradients (7.5–45% [w:w]) in 20 mM Tris-HCl (pH 7.4) including 2 mM magnesium chloride prepared in SW40 clear centrifuge tubes (Beckman Coulter). Centrifugation was for 2 h at 1.5×10^5 *g* at 4 °C. Two turbid bands were visually identified with the more intense one in the middle of the tube and a less intense one in the upper third of the tube. This latter one was brownish, which originates from cellular ferritin as identified by mass spectrometry (data not shown). Both bands were recovered separately with a syringe by perforating the tube. After dilution with 20 mM Tris-HCl (pH 7.4) including 2 mM magnesium chloride, virus was pelleted at 1.0×10^5 *g* overnight at 4 °C. The pellet was suspended in 150 µL 50 mM Tris-HCl (pH 7.4) including 25 mM sodium chloride and aliquots were frozen at −80 °C. The same procedure was employed for the purification of several other viral serotypes. It is of note that for some RV strains an increase in ionic strength in the buffer used for zonal centrifugation was necessary to prevent virus aggregation (Kim et al., 1989). Therefore, the protocol, as given here for RV-A2, probably needs to be modified to suit different virus strains.