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Research Article

Specific ion effects on the particle size distributions of cell culture–derived influenza A virus particles within the Hofmeister series

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Virus particle (VP) aggregation can have serious implications on clinical safety and efficacy of virus-based therapeutics. Typically, VP are suspended in buffers to establish defined product properties. Salts used to achieve these properties show specific effects in chemical and biological systems in a reoccurring trend known as Hofmeister series (HS). Hofmeister series effects are ubiquitous and can affect colloidal particle systems. In this study, influences of different ions (anions: SO_4^{2-} , HPO_4^{2-} , Cl^- , Br^- , NO_3^- , I^- ; cations: K^+ , Na^+ , Li^+ , Mg^{2+} , Ca^{2+}) on particle size distributions of cell culture-derived influenza VP were investigated. For the experimental setup, influenza virus A/Puerto Rico/8/34 (H1N1) VP produced in adherent and suspension Madin Darby canine kidney cells were used. Inactivated and concentrated virus harvests were dialyzed against buffers containing the ions of interest, followed by differential centrifugal sedimentation to measure particle size distributions. VP from both cell lines showed no aggregation over a wide range of buffers containing different salts in concentrations ≥ 60 mM. However, when dialyzed to low salt or Ca^{2+} buffers, VP produced in adherent cells showed increased aggregation compared to VP produced in suspension cells. Additionally, changes in VP diameters depending on specific ion concentrations were observed that partially reflected the HS trend.

Keywords: Hofmeister series / Specific ion effects / Vaccines / Viral vectors / Virus aggregation

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1 Introduction

Virus particle (VP) aggregation is an important factor to be considered for the optimization of the production of viral

vaccines, i.e., VP quantification [1], *in vitro* infectivity assays [2], inactivation of VP [3–5], and downstream processing (DSP) [6]. Accordingly, the characterization of media and buffer components affecting the virus particle size distribution (PSD) is of great interest. Previous investigations used complex methods to analyze virus PSD. For example, pioneering work in this field used a “single particle approximation” (SPA) method employing high-speed centrifugation for separating monomeric VP from multimers to investigate VP aggregation by diluting polio- and reoviruses in water [7,8], the survival of such aggregates [3], the effects of certain salts on VP aggregation at low pH [9], and the occurrence of mixed aggregates of both VP species [10]. The VP separation via SPA was achieved by stopping the centrifugation at a certain time point followed by analysis of the different layers of the centrifuged sucrose gradient fluid by plaque titration or electron microscopy (EM). Furthermore, influenza VP aggregation has been investigated with a modified SPA method in combination with EM to determine the VP content of influenza vaccines [1]. Results showed that all analyzed vaccines contained

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Abbreviations: A/PR, A/Puerto Rico/8/1934; AHD, apparent hydrodynamic diameter maxima; AUC, analytical ultracentrifugation; CCC, critical coagulation concentration; CFV, concentrated and filtered virus samples; CSC, critical stabilization concentration; CVH, clarified virus harvests; DCS, differential centrifugal sedimentation; EM, electron microscopy; GB, gradient buffer; HA, hemagglutinin/hemagglutination; HAU, hemagglutination units; HS, Hofmeister series; MDCK, Madin Darby canine kidney; MWCO, molecular weight cut-off; NA, neuraminidase; PBS, phosphate buffer saline; PSD, particle size distribution; RT, room temperature; SB, standard buffer; SPA, single particle approximation; VP, virus particle

VP aggregates to some degree, which could be dispersed by suspension in high molality buffers. More recent experimental studies explored the effects of certain hemagglutinin (HA) and neuraminidase (NA) gene combinations on the aggregation status of influenza VP by centrifugation in a sucrose gradient followed by analyzing the different layers of the centrifuged fluid by a HA assay [11]. Furthermore, pH-induced aggregation of influenza VP was studied by static and dynamic light scattering [12], and UV absorption [13]. Unfortunately, none of these methods give detailed PSD of the VP and their aggregates [14], which are needed to better understand the involved effects. In addition, size distribution analysis of influenza VP using size exclusion chromatography was performed [15], but not compared to other high-resolution PSD measurement methods.

An established method to obtain high-resolution size distributions of colloids is analytical ultracentrifugation (AUC) [16]. High-end AUC equipment involves substantial costs for equipment and operation and therefore makes it only suitable for specialized research taking full advantage of its features. Since several years, however, comparatively simple centrifuges for particle size analysis are available that use limited rotor speeds and fixed single wavelength detection systems [16]. One example is the CPS DC24000 UHR disc centrifuge from CPS Instruments Inc. (LA, USA) employing differential centrifugal sedimentation (DCS) [17]. The implemented DCS method determines the sedimentation times of the particles of interest with unknown size but known buoyant density in a gradient fluid with known density to calculate their sizes based on Stokes' law [17]. Applications reported so far included the characterization of PSD of recombinant adeno VP [18, 19], L1 virus-like particles [20], and influenza VP [21]. In addition, it was shown that a DCS method enables the determination of PSD with a higher resolution than other dynamic light scattering techniques [14].

The experimental setup in the present work is based on a DCS disc centrifugation method to investigate specific ion effects within the HS on influenza virus PSD. The order of the direct HS is $\text{SO}_4^{2-} < \text{HPO}_4^{2-} < \text{Cl}^- < \text{Br}^- < \text{NO}_3^- < \text{I}^-$ for the anions and $\text{K}^+ < \text{Na}^+ < \text{Li}^+ < \text{Mg}^{2+} < \text{Ca}^{2+}$ for the cations and the indirect HS has the reverse order [22–24]. The ions are ordered according to their effect on the solubility of proteins: early members of the direct HS series increase the solvent surface tension and decrease the solubility of nonpolar molecules ("salting out," kosmotropic), whereas the latter salts increase the solubility of nonpolar molecules ("salting in," chaotropic) [24, 25]. In addition, HS effects on synthetic nanoparticles were described in the literature [24, 26–31]. For biological nanoparticles like VP, however, no systematic evaluations of HS effects by high-resolution PSD measurement techniques have been carried out so far. Therefore, the present work delivers fundamental information regarding the effects of certain HS ions on the PSD of cell culture–derived influenza VP by DCS disc centrifugation.

2 Materials and methods

2.1 Buffer and media solution preparation

Ultrapure water from a Milli-Q® Advantage A10 water purification system from Merck KGaA (Darmstadt, Germany) was used

for all buffers and media solutions. All chemicals used for HS buffers had a purity of $\geq 99\%$ and were obtained from Sigma-Aldrich Co. LLC. (St. Louis, USA) unless otherwise stated; all chemicals used for cell culture media were cell culture media grade. The salts for the HS buffers used for screening were Na_2SO_4 (#S9627), Na_2HPO_4 (#S8282), NaH_2PO_4 (#S7907), NaCl (#S7653), NaBr (#71329), NaNO_3 (#S8170), and NaI (#793558) for the anions, and CaCl_2 (#C5080), KCl (#P9333), LiCl (#62476), and MgCl_2 (#M2670) for the cations. Na^+ or Cl^- salts were used as they are considered reference points in the HS [27]. The concentrations used for the screening were 20, 60, 540 mM for all HS salts, and for NaCl additionally 180, 1020, and 1500 mM. All HS ion concentrations in the HS buffers were based on the specific ion of interest without adjusting the HS buffer recipe for the counterions. For the preparation of all HS buffers, a minimal 10 mM Tris–HCl standard buffer (SB) system was used to maintain the pH at 7.4. All HS buffers were prepared and used at room temperature (RT; 25°C) to avoid pH shifts due to the high temperature dependency of the Tris pKa [32]. For the preparation of 500 mL HS buffer, 0.605 g Tris base (#T6791) and the amount of the HS salt of interest were dissolved in approximately 450 mL ultrapure water, followed by subsequent titration to pH 7.4 with HCl (#100317; Merck KGaA, Darmstadt, Germany) diluted in ultrapure water and fill up to 500 mL with ultrapure water. For the $\text{Na}^+/\text{PO}_4^{3-}$ buffer, the acidic and basic compounds (NaH_2PO_4 and Na_2HPO_4) were mixed to result in pH 7.4 after addition of ultrapure water followed by addition of Tris-base, titration with HCl to pH 7.4, and fill up to 500 mL with ultrapure water. Additionally, NaN_3 (#71290) was added to a final concentration of 0.05% to the HS buffers to inhibit microbial growth. In addition, HS buffers were filtered with a 0.2- μm bottle top filter (#514-0340, VWR, Radnor, USA) to remove particulate impurities. HS buffers were stored at 4°C and were allowed to warm up to RT before usage.

2.2 Hemagglutination assay for virus quantification

The hemagglutination (HA) assay was based on Kalbfuss et al. [33]. Briefly, 100 μL VP sample was serially diluted in round-bottomed 96-well microtiter plates with 100 μL 1 $\times$ phosphate buffer saline (1 $\times$ PBS). For the quantification of the hemagglutination units (HAU), an in-house generated internal influenza A/PR standard was used. Then, 100 μL of purified chicken erythrocytes was added to each well and plates were incubated at RT for 2 h. After that, light extinction was measured at a wavelength of 700 nm and data was evaluated according to Kalbfuss et al. [33].

2.3 Influenza virus particle production

Influenza VP were produced in a suspension (MDCK_{SUS2}) and an adherent Madin Darby canine kidney (MDCK) cell line (MDCK_{ADH}, ECACC No. 84121903) by infection with an in-house generated adherent MDCK-derived A/Puerto Rico/8/34 (A/PR, H1N1) virus seed (#3138, Robert Koch Institute, Berlin, Germany) with a 50% tissue culture infective dose (TCID₅₀) of 1.23×10^8 infectious VP/mL [34–36].

MDCK_{SUS2} cells were cultivated in chemically defined, protein- and peptide-free SMIF8 PGd medium (used 2× concentrated, #M008-2b, Service Zellkultur Scharfenberg, Emden, Germany). Additionally, this medium contained 4 mM L-glutamine, 4 mM pyruvate, 5 g/L NaCl, 3.66 g/L D-(+)-glucose, 2 g/L NaHCO₃, 0.242 g/L L-glutamic acid, 10 mL/L 10% Pluronic-F68, and 1 μL/L 98% ethanolamine. For virus infection, 10⁻⁵ trypsin units/cell (#27250-018, from porcine pancreas, Thermo Fisher Scientific, MA, USA) was added. Cultivations were carried out in a 5-L CT5-SK bioreactor from Sartorius Stedim Biotech GmbH (Göttingen, Germany). For virus production, a multiplicity of infection (MOI) of 10⁻⁴ was used, and the virus broth was harvested 72 h postinfection (hpi). VP produced by MDCK_{SUS2} are subsequently termed as “A/PR_{SUS}.”

MDCK_{ADH} cells were cultivated in GMEM-BHK21 medium (#22100-093, Thermo Fisher Scientific, MA, USA). Additionally, the medium was supplemented with 1 g/L D-(+)-glucose, 4 g/L NaHCO₃, 10% fetal calf serum (FCS; #F7524, Sigma-Aldrich Co. LLC., St. Louis, USA), 0.2% lab-FMV-peptone (#MC033, Lab M Ltd., Heywood, UK). For virus production, cells were washed three times with 1× PBS and fresh medium without FCS and with 5 × 10⁵ trypsin units/mL was added. The VP production was carried out in 850 cm² roller bottles (#680160, Greiner Bio One International GmbH, Frickenhausen, Germany) with 0.5 mL of the in-house generated A/PR virus stock using a MOI of 10⁻⁴. The virus broth was harvested 72 hpi. VP produced by the MDCK_{ADH} system are subsequently termed as “A/PR_{ADH}.”

2.4 Virus broth clarification and inactivation

Harvested virus broths were filtered through a 5-μm depth filter followed by a 0.65-μm depth filter (#CFAP0508YY and #CFAP9608YY, GE Water & Process Technologies, Trevose, USA). After clarification, chemical inactivation with β-propiolactone (#33672.01, Serva Electrophoresis, Heidelberg, Germany) at a final concentration of 6 mM was carried out. The inactivation process was stopped after incubation at 37°C for 24 h and completed by a filtration through a 0.45-μm depth filter (#CMMP9408YY, GE Water & Process Technologies, Trevose, USA) to obtain the clarified virus harvests (CVH).

2.5 Concentration of the clarified virus harvests by tangential flow filtration

For concentrating both CVH to approximately 10⁵ HAU/mL, a Sartocon Slice 200 Hydrosart tangential flow filtration (TFF) cassette (cellulose membrane), kindly provided by Sartorius Stedim Biotech GmbH (Göttingen, Germany) with a molecular weight cut-off (MWCO) of 750 kDa, was used. The concentration via TFF was carried out on an ÄKTAcrossflow TFF system from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). After concentration, both CVH were filtered through a 0.1-μm filter (#SLVV033RS, Merck KGaA, Darmstadt, Germany), aliquoted, and frozen at -80°C to obtain concentrated and filtered virus samples (CFV). The final filtration step ensured that VP aggregates are only newly formed from monomeric VP in the course of the subsequent experiments.

The A/PR_{SUS} CVH was 10× concentrated by volume reduction to obtain the A/PR_{SUS} CFV with 9.6×10^4 HAU/mL after 0.1 μm filtration, aliquotation, freezing (-80°C), and thawing. Using the same approach, A/PR_{ADH} CVH was 40× concentrated to A/PR_{ADH} CFV with 1.3×10^5 HAU/mL. Because of the small difference in the concentrations, the higher concentrated A/PR_{ADH} CFV sample was diluted 1:1.4 in 1× PBS before the subsequent dialysis step (see section 2.6) to a concentration of 10⁵ HAU/mL. No VP aggregation was observed after the described sample preparation (data not shown).

2.6 Buffer exchange via dialysis

Dialysis was used to change the buffer of the CFV samples from cell culture media to the HS buffer of interest. Therefore, 500 μL CFV sample was pipetted into a cellulose dialysis tube with a MWCO of 14 kDa (#0653.1, Carl Roth GmbH & Co. KG, Karlsruhe, Germany) and dialyzed against 500 mL HS buffer with a dialysis factor of 1:10³ under stirring for 24 h at RT. A 14-kDa MWCO dialysis membrane was used to avoid any potential impact of sample concentration during the buffer exchange step. In particular, dialysis membranes with higher cut-offs could induce aggregation because of volume reduction due to high osmotic pressures of higher concentrated HS buffers (data not shown).

2.7 Particle size distribution measurements by differential centrifugal sedimentation

A DCS method was used for the measuring the PSD of the dialyzed CFV samples [17]. The used method was based on Neumann et al. [21], with additional modifications of the setup as described below.

A CPS DC24000 UHR disc centrifuge (CPS Instruments Inc., LA, USA) was used for measuring the virus PSD at a maximum disc speed of 24 000 revolutions per minute (RPM). The gradient buffer (GB, 4–16% (w/v)) was prepared in the respective HS buffer using sucrose (#1.07654, Merck KGaA, Darmstadt, Germany). Therefore, 16, 14.5, 13, 11.5, 10, 8.5, 7, 5.5, and 4% w/v 1.6 mL GB steps with a total GB volume of 14.4 mL were injected. To reduce the risk of conformational changes of proteins adsorbed to dodecane-in-water emulsion interfaces, no dodecane cap was added to prevent GB evaporation [37,38]. After building up the GB, 100 μL of a 239-nm particle standard (0.3–0.5% solid content, polyvinyl chloride (PVC), particle density $\rho_p = 1.385$ g/cm³, CPS Instruments Inc., LA, USA) diluted 1:4.25 in HS buffer was injected to assess the gradient quality. (Note: injection of this particle standard not only ensured GB functionality but also increased gradient lifetime by reducing evaporation at the gradient top surface.) Next, the gradient was equilibrated 10 min followed by another injection of the 239-nm particle standard for calibration (100 μL diluted 1:4.25 in HS buffer). Then, 100 μL of the dialyzed CFV sample was injected for the measurement. After each measurement, the centrifuge disc was cleaned on the in- and outside with warm water to remove any residual gradient solution, and finally wiped out with pure isopropanol.

2.8 Gradient buffer and virus particle density measurements by differential centrifugal sedimentation

To determine the analysis parameters for the proprietary software of the CPS DC24000 UHR disc centrifuge, a density measurement method for deriving the GB density ρ_{GB} and the VP buoyant density ρ_{VP} , or—in general—the particle buoyant density ρ_p , was used [21]. Thus, for determining ρ_{GB} and ρ_{VP} , 100 μ L of the VP sample was spiked with a 105-nm particle standard (final dilution of 1:8 in 100 μ L sample, 5% w/w solid content, poly(methyl methacrylate) (PMMA), particle density $\rho_p = 1.19$ g/cm³, #PMMA-R-KM215, Microparticles GmbH, Berlin, Germany) and a 239-nm particle standard (final dilution of 1:16 in 100 μ L sample, 0.3–0.5% w/w solid content, PVC, particle density $\rho_p = 1.385$ g/cm³, CPS Instruments Inc., LA, USA). The spiked VP samples were measured in triplicate in a 4–16% w/v and an 8–20% w/v sucrose GB.

The ρ_{GB} can be determined by a linear regression based on the measured sedimentation times (t_{Std}), the known densities (ρ_{Std}), and the hydrodynamic diameters (D_{Std}) of the standard particles (see Eqs. (1)–(3) in the Supporting Information). (Note: ρ_{GB} can also be determined by measuring the weight of a defined volume of GB according to the setup of Neumann et al. [21]. However, measuring ρ_{GB} in the DCS setup itself using the applied method will lead to more accurate values. With our setup the fluid of the standard particle injection on top of the GB, the GB evaporation over time, and the slightly different GB viscosities inside the warmed-up centrifuge disc are contributing to the ρ_{GB} determination. In addition, the GB below the detector beam does not contribute to the determined sedimentation times and to the derived ρ_{GB} [39].)

For the determination of the ρ_{VP} , the GB density of the 4–16% w/v ($\rho_{GB4-16\%}$) and the 8–20% w/v ($\rho_{GB8-20\%}$) sucrose GB are determined by linear regression based on the sedimentation times $t_{Std105nm}$ and $t_{Std239nm}$ of the standard particles (see Eqs. (4)–(6) in the Supporting Information). Then, the ρ_{VP} can be derived from the sedimentation times $t_{Std105nm}$ of the 105-nm standard particle and the sedimentation time t_{VP} of the VP by an

additional linear regression. For the determination of ρ_{VP} , the 105-nm particle standard was used as it is closer to the VP size and buoyant density. The ρ_{VP} was determined for A/PR_{SUS} and A/PR_{ADH} in GB buffers made of 1× PBS. (Note: linear regression analysis was based on previous work from Neumann et al. [21] and is explained in detail in the Supporting Information.)

3 Results and discussion

To measure the specific ion effects of the HS buffers on the virus PSD, a two-stage experimental setup was carried out. First, the VP buoyant densities ρ_{VP} for the two VP samples were determined. Second, the prepared VP samples were dialyzed to different HS buffers and their PSD were analyzed.

3.1 Virus particle density measurements by differential centrifugal sedimentation

The VP buoyant density ρ_{VP} determined by regression analysis of the sedimentation times in two different GB was 1.17 g/cm³ for A/PR_{SUS} and 1.18 g/cm³ for A/PR_{ADH}, respectively (data not shown). Both values are similar to reported values for MDCK cell culture–derived influenza A virus strains, i.e., 1.18 and 1.19 g/cm³ measured by the same method [21]. An example of the sedimentation time differences in two different GB (4–16% and 8–20% sucrose) is shown in Fig. 1A for A/PR_{ADH} spiked with the 105- and 239-nm particle standard. The 239-nm particle standard has a retention time of approximately 15 s, whereas the 105-nm particle standard has a retention time of about 190 s for the monomeric peak in the GB. The 105-nm particle standard also shows some aggregation with additional peaks located on the left of the monomeric peak in the range of 50–150 s. Influenza VP are showing the highest retention time in the GB of about 340 s.

Figure 1. (A) Measurement of the time differences for differential centrifugal sedimentation of adherent cell culture-derived influenza A virus particles (A/PR_{ADH}) spiked with a 105- and 239-nm particle standard to determine the virus particle density. (Note: the 105-nm particle standard shows aggregation up to tetramers. Peak identity was confirmed by separate injection of the sample and the particle standards.) (B) Size distributions of suspension cell culture-derived influenza A virus particles (A/PR_{SUS}) dialyzed against standard buffer (SB, 10 mM Tris–HCl pH 7.4) and SB with 20, 60, 180, 540, 1020, and 1500 mM NaCl.

3.2 Specific ion effects on the virus particle size distributions

The screened HS buffers for A/PR_{SUS} were standard buffer (SB, 10 mM Tris–HCl pH 7.4), SB including 20, 60, and 540 mM of all HS ions as well as SB with 180, 1020, and 1500 mM NaCl. A/PR_{ADH} was screened in SB, SB with 20 mM NaCl, as well as SB with 20, 60, and 540 mM Ca²⁺. The HS ion screening for concentrations higher than 540 mM was not possible for all buffers, as density differences between the VP and the GB were not sufficient for the measurements (data not shown). In the following section, the average apparent hydrodynamic diameter maxima (AHD) of the monomeric or multimeric VP (*n* = 3) is reported.

The virus PSD for A/PR_{SUS} in SB with different concentrations of NaCl are shown in Fig. 1B. Overall, AHD increased with higher NaCl concentrations (*p* < 0.05, two-sample *F*-test and *T*-test between neighboring AHD), except for 540 and 1020 mM (*p* > 0.05, details shown in Supplement Table 1 in the Supporting Information). In addition, the VP in SB with different NaCl concentrations just showed a minor dimer peak between 100 and 110 nm. Only the PSD obtained in SB was different with high levels of dimers, trimers, and even tetramers. For the measured multimer pattern, the predicted AHD of the aggregates based on deformable spheres agree well to theoretical values ($D_{app,i}$, *i* = number of particles in the aggregate) monomeric VP (D_{VP}) = 81 nm; dimer = 101 nm ($D_{VP:Dapp,2}$ = 1:1.25); trimer = 115 nm ($D_{VP:Dapp,3}$ = 1:1.42) [40]. The suitability of the prediction based on deformable spheres can be explained by the deformability of influenza A VP shown in previous AUC studies [41]. However, for other measurements carried out in this study, the agreement between the measured and the theoretical AHD was sometimes better approximated by a rigid sphere model or both models, i.e., deformable and rigid spheres, failed to describe the measured AHD (data not shown). Comparable VP aggregation in low

salt buffers and water were reported before for influenza A/B, polio-, and reovirus by EM where large aggregates consisting of hundreds of VP were found [1, 8]. However, the large aggregates are most likely not due to aggregation behavior differences but an artifact of the sample preparation for EM. For the experimental setup and the analytical method used in our study, the formation of such large aggregates is not to be expected.

Particle aggregation is often described by a so-called critical coagulation concentration (CCC) and a critical stabilization concentration (CSC) [24, 26–28]. These two concentrations are separating the slow or reaction controlled regime from the fast or diffusion controlled regime [24, 27]. Furthermore, it is known that aggregation of weakly charged particles is fast in high salt concentrations and highly charged particles aggregate slowly in high salt concentrations [24]. Additionally, the CCC decreases with increasing counterion valence, which is known as the Schulze–Hardy rule [24]. Taken together, it has to be expected that the investigated A/PR_{SUS} VP are highly charged and have a CSC below 20 mM NaCl. Moreover, such low CSC values indicate a high hydrophilicity of the investigated VP [27].

Remarkably, all measured PSD with visible aggregates still showed a monomeric VP peak that indicates incomplete aggregation to multimers. One explanation for that could be a very slow aggregation process, as PSD were measured after a 24 h incubation. Additionally, one could argue that this is due to the chosen sucrose gradient employed in the measurement setup. Nevertheless, a sucrose gradient was also used in the study of Dunlap et al. who used a SPA method [1], and for the characterization of adenovirus in the study of Shih et al. by a DCS disc centrifugation method [19], respectively. In the latter, an 8–24% sucrose gradient was compared to a conventional AUC method without sucrose gradient and only showed consistent minor reductions of the detected aggregate levels [19]. This suggests that the addition of sucrose is not affecting the measured PSD via DCS disc centrifugation.

Figure 2. Change of the apparent hydrodynamic diameter maximum with increasing salt concentrations for monomeric suspension cell culture-derived influenza A virus particles (A/PR_{SUS}) dialyzed against standard buffer (SB, 10 mM Tris–HCl pH 7.4) with 20, 60, and 540 mM Hofmeister series ion concentration. Monomeric apparent hydrodynamic diameter maxima (AHD) trends are indicated by arrows at the top for easy visualization. Results from SB with MgCl₂, Na₂SO₄, and NaI are not shown because the monomeric ADH were not measurable at 540 mM as differences between the virus particle buoyant density and the gradient buffer density were too small.

Results of the monomeric AHD for A/PR_{SUS} VP are shown in Fig. 2 for 20, 60, and 540 mM HS ion concentration. For NaCl, NaNO₃, and KCl, the AHD significantly increased with increasing HS ion concentration over the measured range ($p < 0.05$, two-sample *F*-test and *T*-test between neighboring AHD). However, AHD differences were not significantly different for LiCl and for the Na⁺/PO₄³⁻ buffer over the measured concentration range ($p > 0.05$, two-sample *F*-test and *T*-test between neighboring AHD). This was also observed between 60 and 540 mM NaBr. The high scattering of the triplicates for SB with 540 mM Na⁺/PO₄³⁻ buffer was most likely due to system instabilities because of the high salt concentration leading to too low differences between the VP buoyant density (ρ_{VP}) and the GB density (ρ_{GB}). Interestingly, the monomeric AHD for CaCl₂ significantly decreased with increasing concentration over the measured range ($p < 0.05$, two-sample *F*-test and *T*-test between neighboring AHD). Therefore, cations as well as anions seem to be involved in the observed AHD changes, as the used Na⁺ and Cl⁻ salts show different behavior depending on their counterion. One ex-

planation for these observable trends could be that an increased ion concentration leads to increased ion adsorption on the VP surface, which in turn leads to a larger size and density of the VP and, accordingly, a larger AHD. However, this explanation does not fit for the Na⁺/PO₄³⁻ buffer, for the NaBr buffer at higher concentrations, and for all concentrations of the CaCl₂ buffer. Other explanations for this phenomenon could be that different buffers induce different VP rigidities that result in different AHD, the presence of ion channels in the membrane of cell-derived VP that change their activity depending on the present HS ion, which leads to changes in VP density and/or size, or different GB interact differently with the VP and the standard particles used for calibration.

The A/PR_{SUS} PSD at a concentration of 20 mM HS ion are shown in Figs. 3A and B. Here, a direct HS trend was observable for cations with Na⁺ < K⁺ < Li⁺ < Mg²⁺ and Ca²⁺ as well as for the halogen anions with Cl⁻ < Br⁻ < I⁻ (differences between neighboring AHD were not significant, however; $p > 0.05$, two-sample *F*-test and *T*-test; details are shown in Supplement

Figure 3. (A) Cations and (B) anions particle size distributions (PSD) of suspension cell culture-derived influenza A virus particles (A/PR_{SUS}) dialyzed against standard buffer (SB, 10 mM Tris-HCl pH 7.4) with 20 mM Hofmeister series (HS) ions. (C) Cations and (D) anions PSD of A/PR_{SUS} dialyzed against SB with 60 mM HS ions. (E) Cations and (F) anions PSD of A/PR_{SUS} dialyzed against SB with 540 mM HS ions. Results from SB with 540 mM MgCl₂, Na₂SO₄, and NaI were not measurable due to the very low differences between the virus particle buoyant density and the gradient buffer density.

Figure 4. (A) Particle size distributions of suspension cell culture-derived influenza A virus particles (A/PR_{SUS}) and (B) adherent cell culture-derived influenza A virus particles (A/PR_{ADH}) dialyzed against standard buffer (SB, 10 mM Tris-HCl pH 7.4) with 20, 60, and 540 mM CaCl₂. (C) Particle size distributions of A/PR_{SUS} and A/PR_{ADH} dialyzed against SB and (D) SB with 20 mM NaCl.

Table 2 in the Supporting Information). In addition, the aggregation behavior was similar for all cations and anions in the size range of 60–240 nm, except for Li⁺ which showed slightly increased aggregate levels from 100 to 220 nm. Moreover, dialysis against SB including 20 mM CaCl₂ resulted in aggregates with an AHD of up to 560 nm shown in detail in Fig. 4A.

Furthermore, the screening of the 60 mM HS ions showed no considerable aggregation in the size range of 60–240 nm visualized in Fig. 3C and 3D, except for SB with 60 mM Ca²⁺ where again aggregates of a size of up to 560 nm were observed (also see Fig. 4A). All monomeric AHD were in a very narrow size window of 5 nm, and no significant differences could be observed ($p > 0.05$, two-sample *F*-test and *T*-test between neighboring AHD; data not shown). Furthermore, the monomeric AHD differed more for anions (Fig. 3D). This is interesting because influenza VP carry a negative net charge at pH 7.4 due to their isoelectric point in the range of 5.0–7.0 [42, 43], which renders this a possible coion interaction.

Results for the SB with 540 mM HS ions are shown in Figs. 3E and F. All PSD are similar compared to SB with 60 mM HS ions (Figs. 3C and D), except that the monomeric AHD range was broader for the cations. Interestingly, with Ca²⁺ < Li⁺ < K⁺ < Na⁺, which is the indirect or reverse HS order, the monomeric AHD increased. Differences between neighboring AHD were small, and only for Li⁺ and Ca²⁺ a significant difference was found ($p < 0.05$, two-sample *F*-test and *T*-test; for further details, see Supplement Table 3 in the Supporting Information). As for the lower concentrations of Ca²⁺, the use of 540 mM Ca²⁺ also resulted in aggregate formation up to a size of 560 nm (Fig. 4A). Overall, this seems to be a specific behavior as the VP

showed no substantial aggregation in the other HS buffers. This high number of different stabilizing ions indicates a high hydrophilicity of the VP [27]. Most likely, the slightly larger effects of cations on the monomeric AHD, especially at a concentration of 20 and 540 mM, can be attributed to the negative net charge of the VP. This is also supported by the negative net charge of the VP [42, 43].

The PSD for SB with 20, 60, and 540 mM CaCl₂ for both samples, i.e., A/PR_{SUS} and A/PR_{ADH}, are shown in Figs. 4A and B. In general, A/PR_{ADH} showed to be more aggregation prone when compared to A/PR_{SUS}. Moreover, for both VP samples a shift to smaller monomeric AHD was observed between the lower concentrations, i.e., 20 and 60 mM Ca²⁺, and 540 mM Ca²⁺. Furthermore, A/PR_{ADH} showed different aggregate size distributions with a large number of aggregates at all screened Ca²⁺ concentrations when compared to A/PR_{SUS}. Here, A/PR_{ADH} in SB with 20 mM Ca²⁺ even showed a potential 16-mer peak at 214 nm. This indicates a low CCC for Ca²⁺, which corresponds to the Schulze–Hardy rule and the increased counterion valence of Ca²⁺. However, for the other tested divalent cation (Mg²⁺) this effect was not observed. Other possible explanations for the Ca²⁺-induced VP aggregation could be the presence of residual host-cell membrane compounds, e.g. cadherins, which are involved in Ca²⁺-dependent adhesion of mammalian cells [44], or a decreased neuroaminidase (NA) activity, which is also known to lead to VP aggregation [11, 45]. Conversely, Ca²⁺ is also known to stabilize hydrophilic colloidal particle systems [27].

A similar aggregation behavior for A/PR_{SUS} and A/PR_{ADH} was observed in SB and a different aggregation behavior in SB with 20 mM NaCl (Figs. 4C and D). For SB the monomeric AHD for

both VP samples was similar, but for SB with 20 mM NaCl the monomeric AHD of A/PR_{ADH} was smaller compared to A/PR_{SUS} (Figs. 4C and D). Additionally, A/PR_{ADH} showed in both cases an increased number of large aggregates compared to A/PR_{SUS}. In particular, this was true for the large difference in SB with 20 mM NaCl. Here, A/PR_{SUS} showed practically no aggregation compared to A/PR_{ADH}, which formed large aggregates consisting of multimers up to tetra- and pentamers (Fig. 4D). An explanation for this aggregation behavior could be differences in the glycosylation of the viral membrane protein HA [45]. Previous studies showed HA glycosylation differences for influenza VP produced in suspension and adherent MDCK host-cell lines [46], which could affect VP hydrophilicities and thus VP aggregation behaviors.

4 Concluding remarks

Specific ions effects regarding the PSD of influenza A/Puerto Rico/8/1934 (A/PR, H1N1) VP produced in a suspension (A/PR_{SUS}) and in an adherent MDCK host cell line (A/PR_{ADH}) were characterized. The experimental setup used a DCS disc centrifugation method which proved to be applicable to measure the virus PSD after dialysis to different HS buffers.

VP derived from both cell lines showed to be aggregation prone in low salt and Ca²⁺ containing buffers. No aggregation was visible for the other screened buffers, i.e., 60 and 540 mM for all HS ions as well as 60, 180, 540, 1020, and 1500 mM for NaCl. Furthermore, an influence of HS buffers on AHD differences of the monomeric VP was shown for the first time that partially reflected the HS trend. In addition, A/PR_{ADH} showed higher aggregation in SB with 20 mM NaCl and Ca²⁺ when compared to A/PR_{SUS}.

Whether this is due to differences in the cultivation media (serum-free vs. serum-containing) or depends on the cell line (MDCK_{SUS2} vs. MDCK_{ADH}) is not clear at the moment. However, regarding the impact of VP aggregate formation on yields in DSP as well as on formulation of vaccines, any switch from adherent to suspension cells or changes in media composition should be carefully evaluated.

Practical application

This work shows the application of a differential centrifugal sedimentation disc centrifugation method for virus particle size distribution (PSD) screenings. The high resolution and the accuracy of the implemented PSD measurement method offered interesting insights in the (aggregation) behavior of influenza A/Puerto Rico/8/34 (H1N1) virus particles in different buffer systems. In addition, it can be used for the characterization of other colloidal particle systems, like vaccine or viral vector formulations. Considering the large impact of virus particle size and virus aggregation on downstream processing, formulation, and blending of virus-based biologicals our results address important issues concerning process optimization and product quality.

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