

Article

Unique Aggregation of Retroviral Particles Pseudotyped with the Delta Variant SARS-CoV-2 Spike Protein

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Abstract: Individuals infected with the SARS-CoV-2 Delta variant, lineage B.1.617.2, exhibit faster initial infection with a higher viral load than prior variants, and pseudotyped viral particles bearing the SARS-CoV-2 Delta variant spike protein induce a faster initial infection rate of target cells compared to those bearing other SARS-CoV-2 variant spikes. Here, we show that pseudotyped viral particles bearing the Delta variant spike form unique aggregates, as evidenced by negative stain and cryogenic electron microscopy (EM), flow cytometry, and nanoparticle tracking analysis. Viral particles pseudotyped with other SARS-CoV-2 spike variants do not show aggregation by any of these criteria. The contribution to infection kinetics of the Delta spike's unique property to aggregate is discussed with respect to recent evidence for collective infection by other viruses. Irrespective of this intriguing possibility, spike-dependent aggregation is a new functional parameter of spike-expressing viral particles to evaluate in future spike protein variants.

Keywords: coronavirus; SARS-CoV-2; pseudotyped viral particle; spike protein; variant; aggregation



Citation: Petersen, J.D.; Lu, J.; Fitzgerald, W.; Zhou, F.; Blank, P.S.; Matthies, D.; Zimmerberg, J. Unique Aggregation of Retroviral Particles Pseudotyped with the Delta Variant SARS-CoV-2 Spike Protein. *Viruses* **2022**, *14*, 1024. <https://doi.org/10.3390/v14051024>

Academic Editor: Yong-Hui Zheng

Received: 14 April 2022

Accepted: 5 May 2022

Published: 11 May 2022

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1. Introduction

Genetic variants of the severe acute respiratory syndrome coronavirus 2, SARS-CoV-2, continue to evolve as the virus circulates worldwide, and each variant holds the potential to evade acquired immunity and re-ignite the COVID-19 pandemic [1]. SARS-CoV-2 is an enveloped RNA virus containing a single stranded, positive-sense genome [2]. Prominent, club-shaped spike glycoproteins (spikes) project from the viral envelope, mediating binding and fusion between the viral envelope and host cell membranes to deliver the viral genome [3,4]. Spikes are highly immunogenic, eliciting a robust neutralizing antibody response; it is the immunogen encoded by the extremely efficacious mRNA vaccines [5,6]. Due to its essential role in viral entry and immunity, mutations occurring on the spike require careful genetic, structural, and functional surveillance.

The fully assembled, prefusion spike consists of a trimer of spike protomers, each of which is highly glycosylated [7,8]. Proteolytic cleavage of the SARS-CoV-2 spike by furin during biosynthesis nicks the spike into two subunits, S1 and S2 [5]. The S1 subunit contains the receptor binding domain (RBD), which can be in an up (receptor accessible) or down (receptor inaccessible) conformation [6], the N-terminal domain (NTD), and two C-termini. S1 caps the S2 subunit which harbors the membrane fusion machinery [5,6,9]. The spike is

highly flexible due to a hinged stalk [10,11], which may facilitate RBD binding to the host cell receptor, angiotensin converting enzyme II (ACE2) [5,9,12]. Once attached to the host cell by ACE2 binding, a second cleavage event occurs in S2 by a host cell protease, either TMPRSS2 at the cell surface or cathepsin in the endosomal membrane, depending on cell type, protease availability, and viral variant [13,14]. Upon S2 cleavage, the fusion peptide undergoes large conformational changes that drive fusion between membranes [15,16].

Millions of SARS-CoV-2 genomic sequences have been cataloged since the original strain emerged in Wuhan, China in December 2019 [17,18]. Amino acid substitutions or deletions in the spike that impart fitness benefits to the virus have evolved and sometimes converged independently in different geographical locations [19,20] giving rise to several variants designated Variants of Concern (VOCs) by the World Health Organization [20,21]. The first was a single amino acid change from an aspartic acid to a glycine at amino acid 614 (D614G) that increased transmissibility [22], possibly by stabilizing the prefusion spike trimer [23], increasing the RBD up/receptor accessible conformation rate [24], and increasing the spike density on the virion [25]. This D614G variant, designated Pango lineage B.1 [26], rapidly supplanted the original Wuhan strain globally [27] by May 2020, and the D614G substitution is present in all subsequent variants [28]. Next, the VOC Alpha (lineage B.1.1.7) emerged in the UK and became dominant worldwide by early 2021, owing to additional substitution mutations occurring in the spike RBD that increased ACE2 receptor affinity, deletions in the NTD that increased immune escape, and a mutation in S2 that may enhance membrane fusion potential [29–31], collectively leading to about two-fold enhanced transmissibility of Alpha compared to other contemporaneously circulating variants [32].

The Alpha variant prevailed until it was outcompeted by the VOC Delta (lineage B.1.617.2), a member of the B.1.617 lineage, that arose in India and became globally dominant in mid-2021 [33]. The Delta variant was considerably more transmissible than Alpha [34], infected individuals faster, produced earlier detection by PCR test with higher viral load [35–37], and was more pathogenic [38]. The Delta variant's spike displayed a different combination of mutations compared to Alpha, that were predicted to reduce sensitivity to neutralizing antibodies [39–41] and increase ACE2 receptor binding, spike stability, viral infectivity, fusogenicity, and pathogenicity [42–44].

Functional consequences of spike mutations can be tested using engineered mimics of enveloped viruses, called pseudotyped viral particles (PVs). PVs are produced by co-transfecting producer cells with plasmids encoding a capsid protein from a parental virus (typically a retrovirus or arbovirus capsid), the spike glycoprotein of interest, and a reporter gene that produces a fluorescent or luminescent protein signal upon host cell entry. The capsid core buds efficiently from the producer cell while incorporating the heterologous spike and encapsulating the reporter gene [45,46].

Recently, studies comparing the entry rate of PVs packaged with various SARS-CoV-2 spike variants into target cells showed that PVs bearing the Delta spike (Delta PVs) drove markedly faster initial infection, and greater infection overall, than other variants [35,43]. This phenotype is similar to real-world reports of faster initial infection by Delta SARS-CoV-2 [37].

To search for an ultrastructural correlate to the increased initial infection rate of Delta PVs, the structures of murine leukemia virus (MLV)-based PVs bearing several different SARS-CoV-2 spike variants were examined by negative stain transmission electron microscopy (TEM): D614G, Alpha, Delta, Delta sublineage AY.4.2 (Delta AY.4.2), and Omicron sublineage BA.1 (Omicron BA.1, lineage B.1.1.529), along with 'Bald PVs', that lacked a spike glycoprotein. Negative stain TEM showed that PVs bearing Delta and Delta AY.4.2 spikes uniquely clustered into aggregates, whereas the other variant spike PVs and Bald PVs did not. There are clear indications of spike tip interactions mediating this aggregation. The aggregation of Delta and Delta AY.4.2 PVs was confirmed by flow cytometry and nanoparticle tracking analysis (NTA). Cryo-electron microscopy of Delta PV aggregates showed spike-tip interactions between PVs as observed by negative staining.

Implications for Delta spike-mediated aggregation for the kinetics of initial viral infection are discussed.

2. Materials and Methods

2.1. Cell Culture and Production of SARS-CoV-2 Variant Spike PVs

PVs were prepared as previously described [45]. In brief, HEK 293T cells were generously provided by Dr. Gary Whittaker (Cornell University, Ithaca, NY, USA). Cells were maintained in DMEM (Genesee Scientific Corporation, El Cajon, CA, USA) with 10% FBS (Corning, Durham, NC, USA), 1X Pen-Strep (Thermo Fisher Scientific, Waltham, MA, USA), and 250 mg/mL G418 (TOKU-E, Bellingham, WA, USA). The day before transfection, eight million HEK 293T cells were plated on a 10 cm cell culture dish (EZ BioResearch, St. Louis, MO, USA) in 16 mL of DMEM with 10% FBS (no antibiotics were added). Three plasmids, pCMV-MLV-gag-pol, pTG-Luc and SARS-CoV-2 spike expression vector, were transfected into HEK 293T cells at the ratio of 3:4:3 using Lipofectamine 3000 transfection reagent (Thermo Fisher Scientific, Waltham, MA, USA). GISAID Accession IDs of the spike proteins used in the study were: D614G (B.1) EPI_ISL_464996; Alpha (B.1.1.7) EPI_ISL_736724; Delta (B.1.617.2) EPI_ISL_2020954; Delta AY.4.2 EPI_ISL_5423152; Omicron BA.1 (B.1.1.529) EPI_ISL_6699757. To increase expression of the spike protein on the cell surface, the C-terminal 19 amino acids were deleted [47,48]. After 48 h, post-transfection, the supernatants were collected and centrifuged at $290 \times g$ for 7 min at 4 °C. The supernatants were then passed through a 0.45 μm syringe-tip filter and stored on ice or 4 °C until use.

2.2. Negative Stain Transmission Electron Microscopy (TEM) and Immunogold Labeling

PVs were stored on ice after harvest from producer cells and processed as follows for TEM within 4 h of harvest or after overnight storage at 4 °C. All reagents were obtained from Electron Microscopy Sciences (EMS, Hatfield, PA, USA) and all steps took place at room temperature unless otherwise specified. PVs, suspended by gentle trituration, were adhered to freshly glow discharged, formvar and carbon-coated, 300-mesh gold EM grids (EMS, Hatfield, PA, USA) by inverting grids for 2 min on 5 μL drops of culture medium supernatant containing PVs. Grids were rinsed by transferring the grids across three droplets of filtered DPBS (Dulbecco's phosphate buffered saline, Mg and Ca-free, Gibco, Paisley, UK). All solutions were filtered through a 0.22 μm syringe filter with RC membrane (EMS, Hatfield, PA, USA) before contact with the EM grid. For negative staining, grids were rinsed twice with filtered, distilled water and then placed on a drop of filtered 1% aqueous uranyl acetate for 1 min, and then blotted to dryness with filter paper (Whatman #1).

To immunogold label the spike protein S1 subunit, grids with adhered PVs were floated on drops of blocking solution containing 2% BSA (Sigma, St. Louis, MO, USA) in DPBS for 10 min. The primary antibody to SARS-CoV-2 spike subunit 1 (Sino Biological, Beijing, China; Cat. No. 40589-T62) was diluted 1:25 in blocking solution and grids were transferred to drops of primary antibody for 30 min. Then, grids were transferred across two drops of blocking solution for 10 min before incubation with 10 nm gold-conjugated donkey- α -rabbit secondary antibody (EMS, Hatfield, PA, USA) diluted 1:20 in blocking solution for 30 min. Grids were covered during incubation steps to prevent evaporation. Finally, grids were rinsed with three drops of DPBS and negative stained as described above. Grids were observed using a FEI Tecnai T20 transmission electron microscope (Thermo Fisher, Waltham, MA, USA) operated at 200 kV, and images were acquired using a NanoSprint1200 CMOS detector (AMT Imaging, Woburn, MA, USA). Images were prepared for display using Photoshop 2022 (Adobe Systems, San Jose, CA, USA). The sizes of the Delta variant PV aggregates were measured using Fiji. A freehand perimeter was traced around each aggregate to obtain area and size measurements.

2.3. Flow Cytometry of PVs

PVs at 4 h, 24 h, and 7 days were labelled with 0.5 μM BODIPY FL Maleimide (B10250 Thermo Fisher, Waltham, MA, USA), a sulfhydryl-reactive hydrophobic dye, in PBS for 15 min at 4 °C. Samples were acquired on a BD Symphony A5 flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) with forward and side scatter in log mode and thresholding set on BODIPY-positive events. MegaMix-Plus SSC microparticles (Diagnostics Stago, Parsippany, NJ, USA) were used to optimize scatter settings and establish size regions. Data was analysed in FlowJo 10.8 (BD Biosciences, San Jose, CA, USA).

2.4. Nanoparticle Tracking Analysis (NTA) of PVs

The size and concentration of PVs at 4 h, 24 h, and 7 days were characterized by NTA using a NanoSight NS300 (Malvern Instruments Ltd., Malvern, UK) equipped with a 405-nm laser. PVs were diluted 1:100 in PBS, loaded into 1 mL syringes, introduced into the sample chamber using a syringe pump, and three 60 s videos were recorded per sample. Acquisition and analysis settings were kept constant for all samples. Size distributions and concentrations were analyzed in NTA software, version 3.4. Aggregate percentages were calculated by summing the area under the size distribution curve for all species greater than the monomer, normalized by the total area under the curve.

2.5. Cryo Electron Microscopy

A total of 7 μL of PVs at 4 or 24 h was adhered to the top side of freshly glow discharged, 200-mesh Quantifoil gold R2/1 EM grids (EMS, Hatfield, PA, USA), back-blotted for 6–8 s after a 30 s pre-blotting time and immediately plunge frozen into liquid ethane using a GP-EM2 (Leica Microsystems, Wetzlar, Germany), with a chamber maintained at 20 °C and 95% humidity. Grids were screened using a FEI Tecnai T20 transmission electron microscope (Thermo Fisher, Waltham, MA, USA) operated at 200 kV. Images were acquired using SerialEM [49] and direct electron detector K2 Summit (Gatan Inc., Pleasanton, CA, USA) at a nominal magnification of 25,000 \times at a binned pixel size of 3.04 Å/px and about $-3\ \mu\text{m}$ defocus. The dose rate was 8 $e^-/\text{px}/\text{s}$ and 10 s exposures with 0.2 s frames were collected. Each 50-frame-movie was motion corrected using SerialEM. To detect the size of the larger Delta variant PV aggregates, a medium magnification of 1700 \times at a binned pixel size of 88.7 Å/px was used.

3. Results

3.1. PVs Bearing the Delta Spike Variant Cluster into Aggregates

Negative stain TEM was conducted to compare the structure of Delta PVs to those bearing other SARS-CoV-2 spike variants: D614G, Alpha, Delta AY.4.2, and Omicron BA.1. As a control, PVs produced in the absence of a spike, referred to as “Bald”, were also examined. For each set of experiments, PVs were produced in parallel, maintained on ice or at 4 °C, handled under identical conditions, and prepared for TEM within 4 h of harvest. By negative stain TEM, all spike variant PVs showed a population of $\sim 120\ \text{nm}$ diameter spherical particles (Figure 1A–D), the expected size of MLV-based particles [50]. PVs were abundant in the culture medium without concentration, producing a density on the surface of the EM grid of about five PVs per 60 μm^2 of EM grid surface. Also uniformly distributed on the grid surface were $\sim 25\ \text{nm}$ lipoprotein-like particles, sometimes closely associated with PVs, including the Bald PVs. Collapsed dehydrated vesicles resembling extracellular vesicles were observed occasionally, but rarely.

Spike variant PVs often displayed a fringe of spike-like projections on a portion of their surface. Immunogold labeling, directed against the S1 subunit, labeled spikes on D614G, Alpha, Delta, and Delta AY.4.2 variant PVs, but did not label the Omicron BA.1 PVs. The negative control Bald PVs that lacked spikes appeared smooth compared to the others and did not immunogold label (Figure 1).

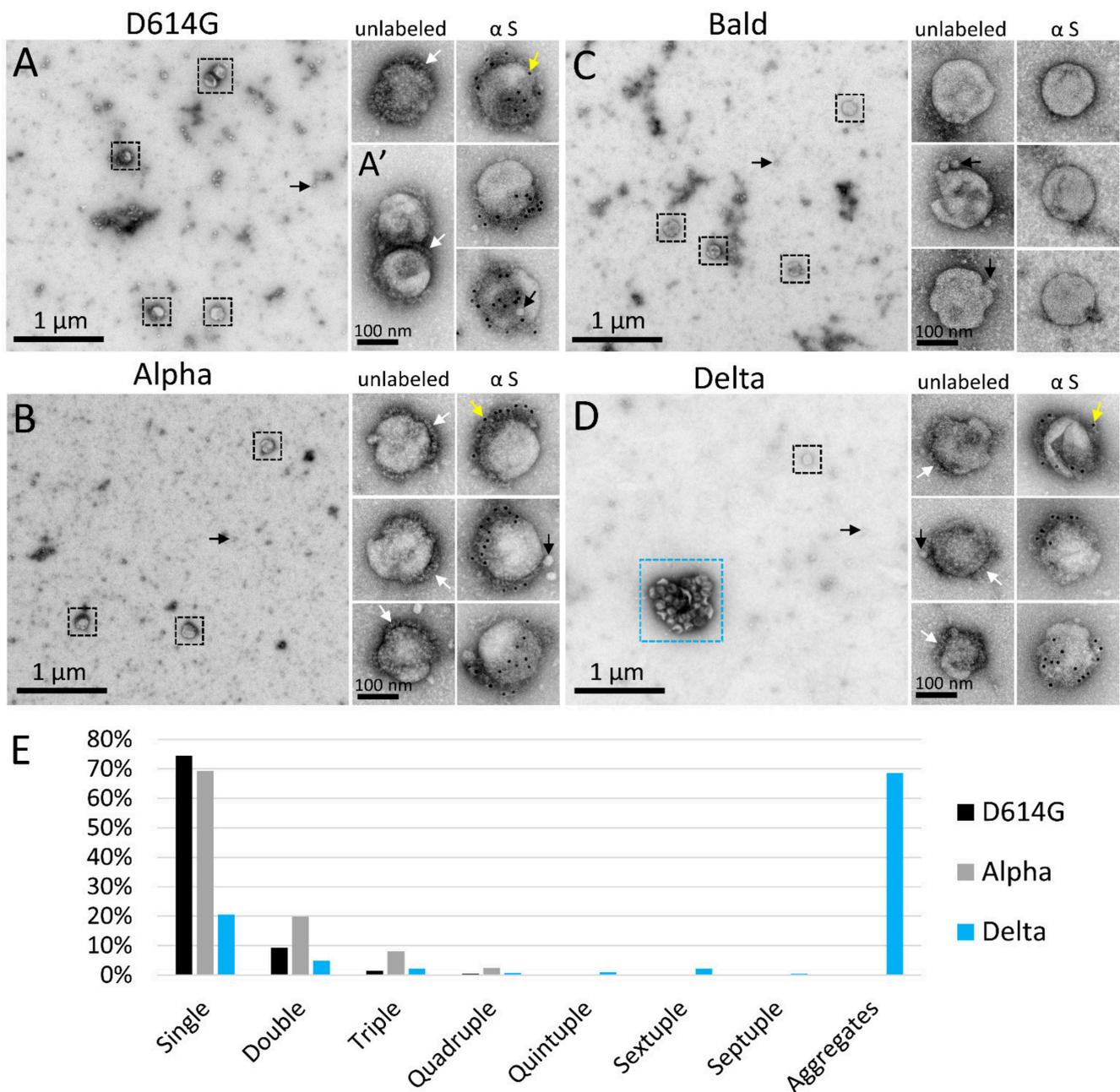


Figure 1. Negative stain TEM detects unique aggregation of PVs bearing the Delta spike. (A–D) Low magnification negative stain TEM overviews of PVs bearing SARS-CoV-2 spike variants (A) D614G, (B) Alpha, (C) Bald, or (D) Delta. Black boxes surround individual PVs; blue box indicates an aggregate of Delta PVs. To the right are enlarged views of individual unlabeled PVs and PVs immunogold labeled for spike protein. (A') Example of a doublet of D614G PVs. White arrowheads show areas on PVs where a fringe of spike proteins is clearly visible. Yellow arrowheads indicate 10 nm immunogold particles labeling the spike S1 subunit, which appear as black dots. Small black arrows indicate lipoprotein-like particles. (E) Frequency of singles, multiples, and aggregates of eight or more PVs per variant.

While the different spike variant PVs looked similar on an individual basis, there was a dramatic difference in the interaction between Delta and Delta AY.4.2 PVs, versus the other variant and Bald PVs. The vast majority of D614G, Alpha, Omicron BA.1, and Bald PVs existed as single PVs or occasionally in groups of 2–3 closely associated PVs (Figure 1A'). In contrast, in three experimental replicates, half or more of the Delta PVs

were sequestered into aggregates of eight or more (up to dozens) PVs (Figures 1D and 2). To quantify the degree of aggregation of the variant PVs, low magnification images were collected at random, and PVs counted and categorized as single PVs spaced more than 60 nm apart (beyond the length of two spike proteins), or in a cluster of two, three, or more PVs. In three experimental replicates, D614G and Alpha PVs were present as single PVs about 75% of the time. Omicron BA.1 PVs (two experimental replicates) were also primarily present as single PVs (not quantified). Bald PVs aggregated at the same rate as D614G and Alpha, suggesting that these spike variants had a comparable and minimal effect on PV interactions that promote aggregation.

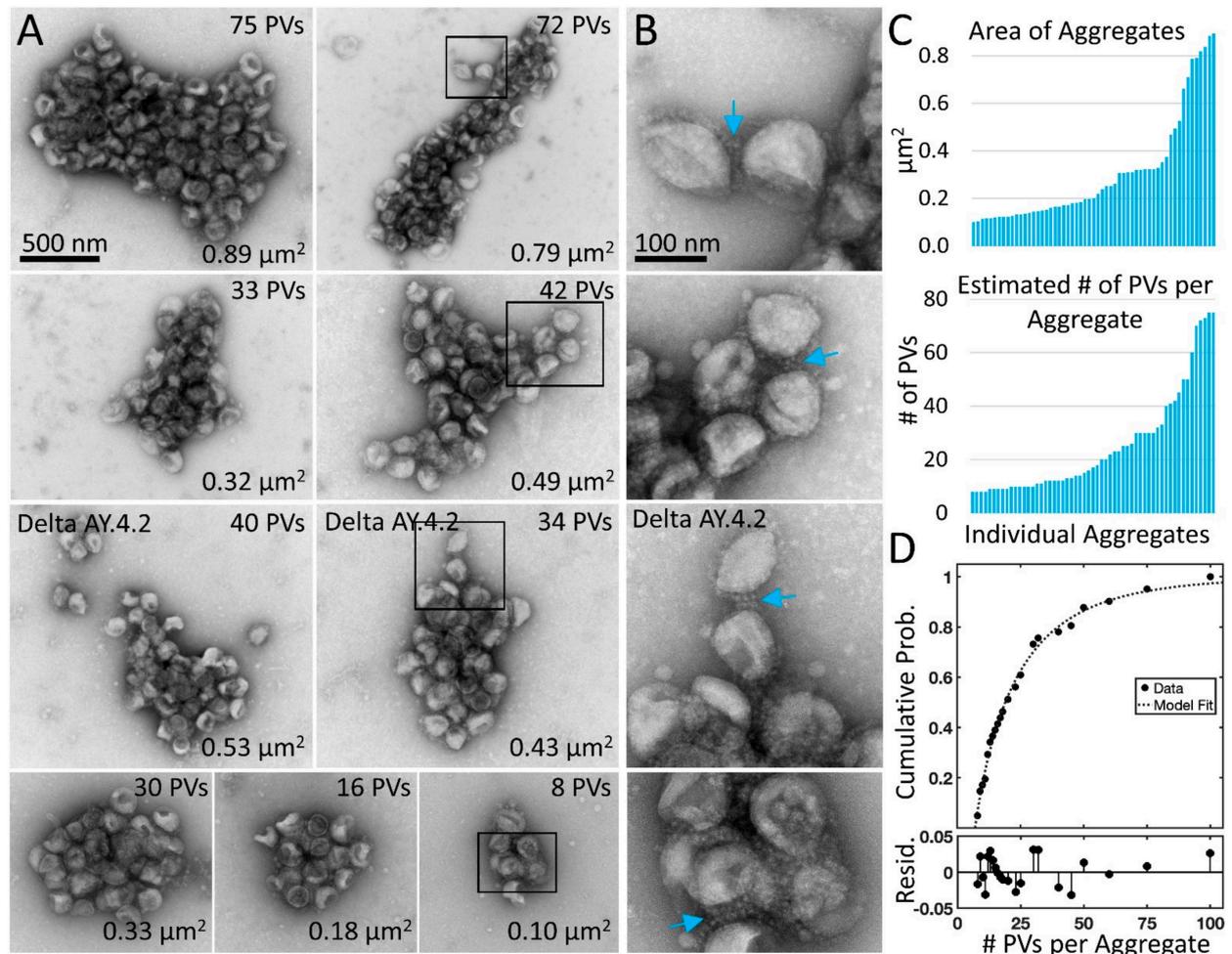


Figure 2. Negative stain TEM of Delta and Delta AY.4.2 aggregates observed 4 h after harvest from producer cells. (A) Delta and Delta AY.4.2 PV aggregates representing range of sizes at the 4 h timepoint. The number of PVs estimated per aggregate is indicated in the upper right corner of each image and the area occupied by the aggregate indicated in the lower right corner. Two Delta AY.4.2 PV aggregates are labeled, all others shown are Delta PVs (unlabeled). (B) Areas boxed in A are shown enlarged to the right of each image. Blue arrows highlight spike tip interactions occurring between PVs at the periphery of aggregates. (C) Graphs show ordered lists of the estimated number of PVs and areas of aggregates. (D) Truncated Weibull Distribution for Delta Variant. Negative stain data at 4 h was fit by the general model: $f(x) = (\text{wblcdf}(x,a,b) - \text{wblcdf}(7,a,b)) / (1 - \text{wblcdf}(7,a,b))$ where wblcdf is the Weibull Distribution and x is the PVs per aggregate, 7 is the truncation value, and a and b are the Weibull scale and shape parameters. Coefficients (with 95% confidence bounds) are $a = 11.47$ (8.35, 14.60) and $b = 0.68$ (0.57, 0.79). Goodness of fit measure Sum of Squared Error (SSE) = 0.009 and Standard Error of Regression (RMSE) = 0.021, and Resid. are the residuals or differences between the model fit and the data.

The average Delta PV aggregate occupied $0.3 \mu\text{m}^2$ and was 700 nm in its longest axis (57 Delta PV aggregates containing eight or more PVs measured in three experimental replicates, average area $0.30 \pm 0.23 \mu\text{m}^2$, average major axis $708 \pm 300 \text{ nm}$) (Figures 1D and 2). The number of PVs in an average-sized aggregate was ~25 PVs, however this is likely an underestimate as PVs inside the aggregate were obscured from view, particularly in the case of larger aggregates. The Delta variant sublineage Delta AY.4.2 exhibited the same aggregation property (one experimental replicate, 48% of Delta AY.4.2 PVs in aggregates of 8 PVs or more).

To gain insights into the interactions between Delta PVs in aggregates, PVs on the periphery of aggregates were examined at high magnification. Examples of Delta PVs with clear indications of spike tip interactions were observed between PVs associated with the edge of aggregates (Figure 2C, white arrows). However, since negative stain requires the PVs to be dried in a thin layer of electron dense stain, it was not possible to see into deeper areas of the aggregate where PVs were layered on top of each other.

From this dataset, the aggregation properties of Delta are different from Alpha and D614G. This conclusion is supported by several lines of evidence. First, whereas Delta exhibits overdispersion, Alpha and D614G exhibit underdispersion. Second, whereas the Alpha and D614G have similar distributions with low probability of aggregates with three or more PVs, Delta has a long tail with higher probability of aggregates with three or more PVs ($>10\times$). Third, Alpha and D614G distributions are described by truncated Poisson distributions with a similar parameter whereas the Delta distribution is better described by a truncated Weibull distribution (Figure 2D).

3.2. Delta Spike Variant PVs Continue to Aggregate in Solution

The same samples of PVs that were prepared within 4 h of harvest were stored at 4°C overnight (still suspended in culture medium) and prepared again for negative stain TEM the next day. PVs of D614G, Alpha, Omicron BA.1, and Bald PVs remained predominantly singles and showed minimal aggregation. However, after overnight storage, the aggregates of Delta and Delta AY.4.2 PVs were much larger, less frequent, and required lengthy searching with the microscope to find. In parallel, there was a concomitant reduction in single Delta and Delta AY.4.2 PVs on the grid surface indicating that single PVs continued to become sequestered in aggregates while diffusing in the culture medium and aggregates themselves likely aggregated. Aggregates at 24 h were irregularly shaped, sometimes donut-shaped, and up to several microns in size (Figure 3).

3.3. Flow Cytometry and NTA Confirm Population of Aggregated Delta Spike Variant PVs

Since negative stain TEM permits observation of only those entities that adhere to the surface of the EM grid, we applied two alternative methods, flow cytometry and nanoparticle tracking analysis (NTA), which sample particles present in the entire solution volume to corroborate the negative stain TEM observations. All variant PVs measured by flow cytometry and NTA within four hours of harvest from producer cells detected particles predominantly in the single PV range (not shown, $n = 2$). However, after 24 h stored at 4°C , flow cytometry and NTA of PVs detected a significant population of larger particles in the Delta PVs that was not present in the other variant PVs or the Bald PVs (Figure 4A,C, $n = 2$). Because NTA does not count aggregates above $1 \mu\text{m}$ in size, the larger aggregates observed by negative stain TEM are not reflected in the NTA analysis. However, since the abundance of single Delta PVs (peak ~150 nm) reduced by half compared to other PVs, it is likely explained by their sequestration into aggregates (Figure 4C). Despite these differences in sensitivity, a significant population of Delta PVs was observed in large aggregates by flow cytometry and NTA that was absent in PVs bearing other variant spikes and Bald PVs. After storage of PVs at 4°C for 7 days, flow cytometry was repeated and still minimal aggregation of D614G, Alpha, or Bald PVs was detected, suggesting that these variants and Bald PVs have less tendency to aggregate under these conditions (Figure 4B,

$n = 2$). However, after 7 days there was evidence for some deterioration in the functionality of PV samples, as cell entry was very low (data not shown).

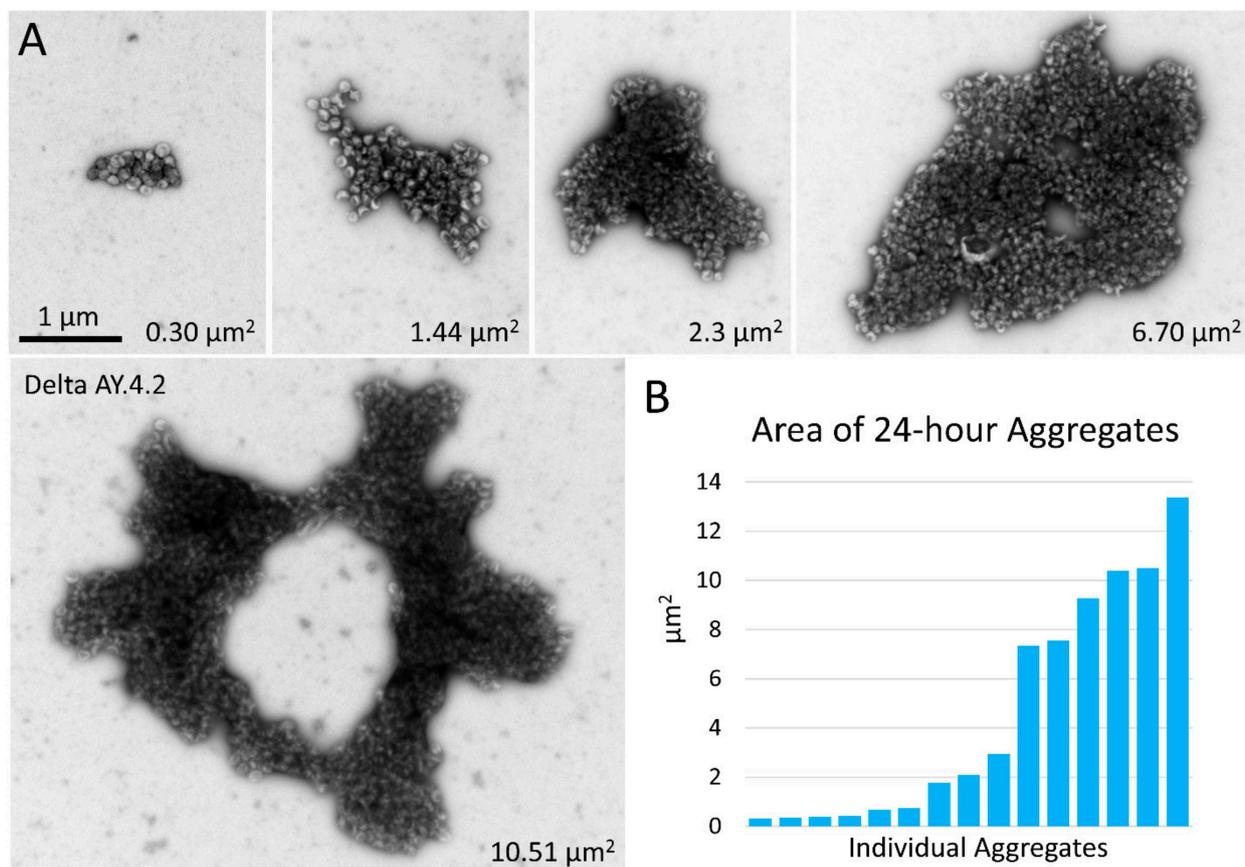


Figure 3. Negative stain TEM of Delta and Delta AY.4.2 aggregates 24 h after harvest from producer cells. (A) Images of aggregates of Delta and Delta AY.4.2 PVs representing a range of sizes observed after storage overnight at 4 °C. The area occupied by each aggregate is indicated in the lower right corner. One donut-shaped Delta AY.4.2 aggregate is labeled, all other aggregates are Delta PVs (unlabeled). All images are scaled the same and scale bar is indicated. (B) Graph shows an ordered list of the areas of Delta aggregates.

3.4. Cryo-Electron Microscopy of Delta Variant PVs

To further evaluate the interactions between spike variant PVs, we used cryo-EM, which preserves PVs in hydrated vitreous ice, free from heavy metal stains and potential artifacts of drying that are inherent to negative stain TEM. Delta and all other variant PVs (4 h and 24 h post-harvest) were adhered to holey grids and plunge frozen. Imaging confirmed the observations from negative staining that PVs harboring D614G, Alpha, or Omicron BA.1 spikes, as well as the Bald PVs without any spikes, existed mostly as single PVs, or in some rare cases, in groups of two to three closely associated PVs. In contrast, Delta and Delta AY.4.2 PVs mostly existed in aggregates (Figure 5A,B). LDL particles near and far from PVs were observed in all samples with a size range between 19–22 nm. Bald vesicles, damaged PVs, and free Gag were also seen on occasion. The log-normal cumulative distributions show the shift expected for time-dependent aggregation (Figure 5C). There is no evidence to support a significant difference in aggregate sizes between Delta and Delta AY.4.2.

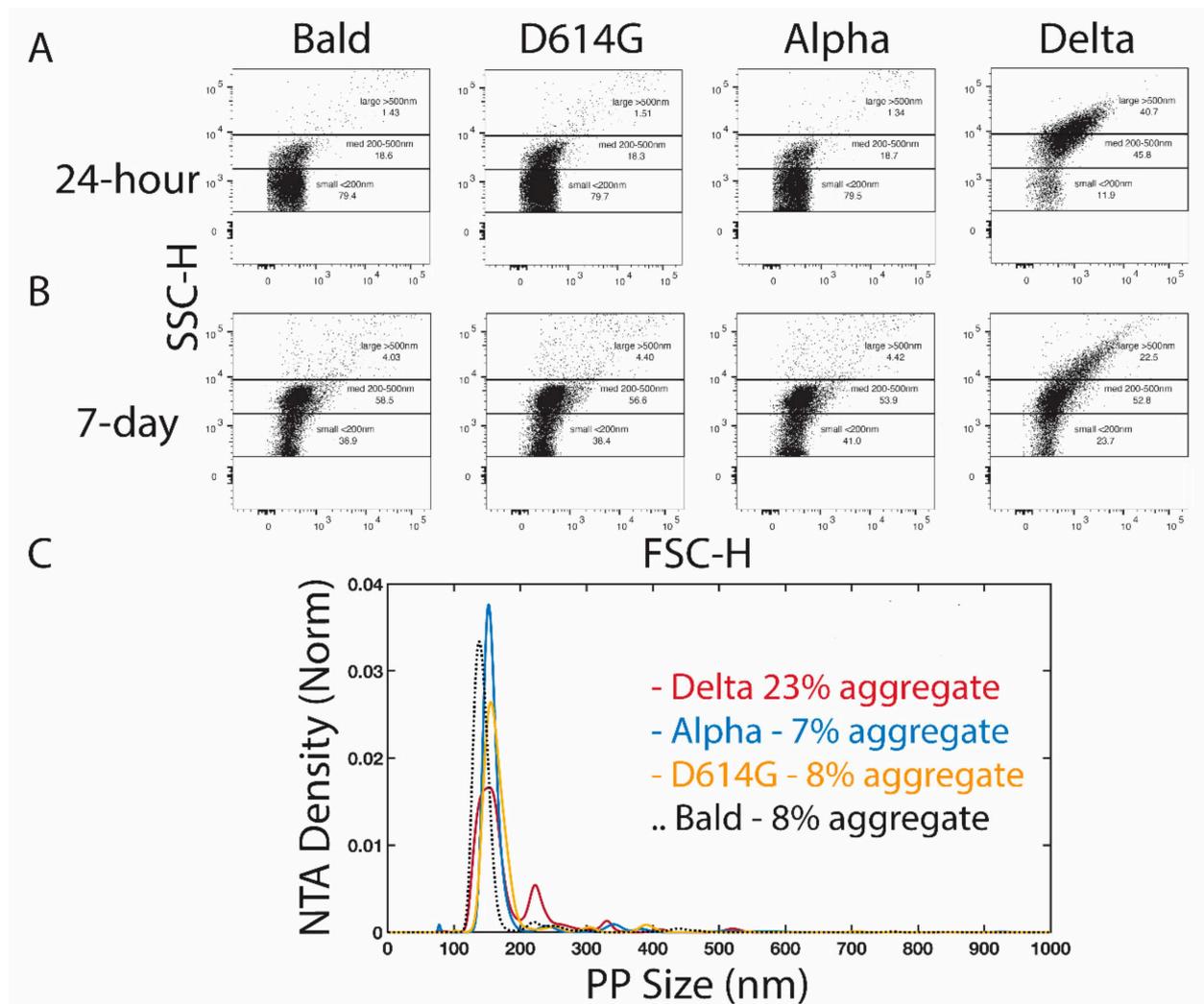


Figure 4. Flow cytometry and NTA detection of Delta PV aggregates. Representative flow cytometry of variant PVs conducted 24 h (A) and 7 days (B) post-harvest. A population of particles over 200 nm in size are present in the Delta PVs and are minimally (<10%) represented in the other PV variant samples. (C) NTA of the same samples of PVs at 24 h post-harvest shows about half as many particles in the single PV range in the Delta sample compared to the other variants, and detection of a larger proportion of PVs in aggregates.

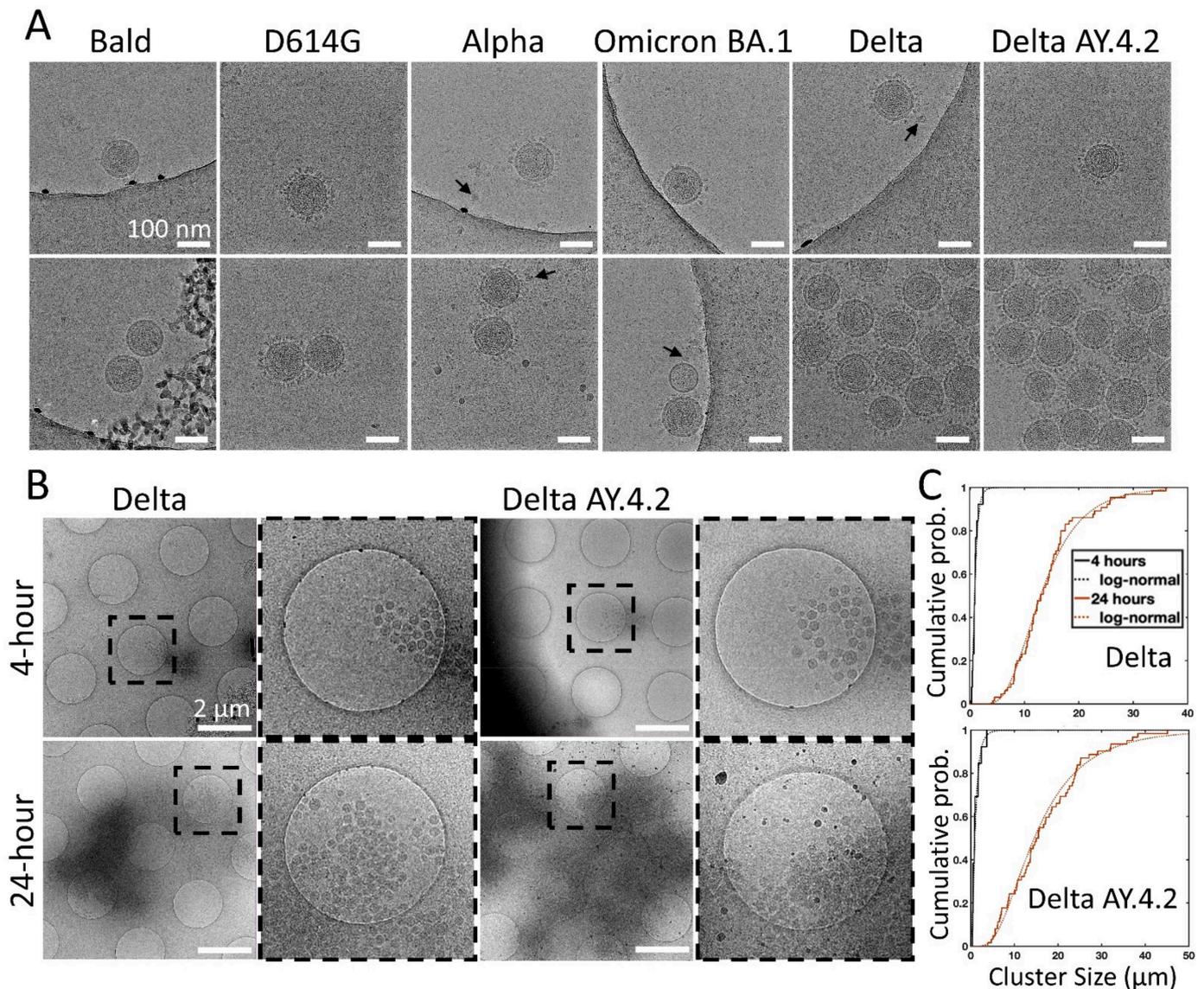


Figure 5. Cryo-electron microscopy of spike variant PVs. (A) Examples of singles (top panel), doubles, or aggregates (lower panel) of each variant PV. Spike variant is identified above. Black arrows indicate lipoprotein-like particles. The average PV envelope diameters for each variant are 118.6 (1.1), 118.5 (1.2), 119.6 (0.9), 120.1 (1.3), 115.8 (0.8), and 118.4 (0.6) nm for Bald, D614G, Alpha, Omicron BA.1, Delta, and Delta AY.4.2, respectively (mean (SEM), $n = 9, 33, 40, 18, 65,$ and 98). (B) Low magnification overviews of Delta and Delta AY.4.2 PV aggregates prepared 4 or 24 h post-harvest. Dashed box shows the zoomed-in area. (C) The log-normal cumulative distributions for Delta and Delta AY.4.2 clearly show aggregation over time for both variants. At 4 and 24 h, Delta aggregate sizes (longest axis) range from 0.28–2.39 and 4.20–36.0 μm , with log-normal parameters μ (σ) -0.18 (0.56) and 2.56 (0.44), corresponding to means of 0.97 and 14.25 μm , respectively. There is no evidence for significant differences in aggregate sizes between Delta and Delta AY.4.2 for both 4 and 24 h.

4. Discussion

We demonstrate, using four independent techniques—negative stain TEM, flow cytometry, NTA, and cryo-EM—that PVs bearing the Delta variant spike or the closely related Delta sublineage Delta AY.4.2 spike aggregate, whereas PVs bearing other spike variants and Bald PVs do not aggregate. Since the PVs were prepared in parallel, handled under identical conditions, and factors that could promote aggregation such as pH changes, freeze-thaw cycles, and high-speed ultracentrifugation were avoided, the observed aggregation of

Delta PVs most likely reflects a unique property of the Delta and Delta AY.4.2 spikes. The observation that Delta and Delta AY.4.2 PVs continued to aggregate in solution while stored at 4 °C suggests that aggregation occurs after budding from the producer cell; however, interaction between PVs could also initiate during biosynthesis and budding from the producer cell.

Unlike SARS-CoV-2, which buds into the ERGIC compartment during final assembly, retroviruses, including MLV, generally bud directly from the plasma membrane (but not always, see [51]). Thus, it is not known when or if Delta variant SARS-CoV-2 aggregates and this is currently being investigated. Delta SARS-CoV-2 could aggregate while budding into the ERGIC, during egress through alkalized lysosomal organelles [52], after egress in the extracellular milieu, or even on the target cell plasma membrane. The ability to aggregate may depend on the concentration of viral particles in each environment. The fact that Delta PVs continue to aggregate while stored at 4 °C (Figures 3–5) is consistent with a mass action mechanism for Delta PV aggregation. Measurements of the rate of aggregation of Delta PVs at temperatures ranging up to physiological temperature could shed light on the thermodynamic properties of Delta aggregation, and advance understanding of the mechanism of aggregation. Furthermore, to produce PVs, a 19 aa C-terminal truncated version of each variant spike was expressed, which has been shown to increase the amount of spike incorporated into the PV envelope and PV infectivity [47,48]. Truncations in the cytoplasmic tail could modify properties of the spike ectodomain structure and function [53–55]. Though each variant spike possessed the same truncation, it is not impossible that the truncation uniquely affected the Delta spike ectodomain, conferring the aggregation property.

Examples of Delta PVs with apparent spike tip interactions were observed by negative staining but these interactions did not extend to lateral aggregation of spike proteins on the surface of a PV. Ongoing cryo-electron tomography studies will reveal the nature of the interactions between aggregated PVs.

Spike-mediated aggregation differs from antibody-driven aggregation of virions expected from polyvalent neutralizing antibodies, as those binding constants are expected to be much stronger. Thus, virions would be tightly packed and not likely to disaggregate at the cell surface. The spike tip interactions are more likely to come apart upon surface binding and receptor competition for the RBD at the tip of the spike.

Analysis of the mutations present in the Delta and Delta AY.4.2 spikes compared to other variants may provide clues as to their unique property to aggregate. Delta and Delta AY.4.2 amino acid sequences are similar, except for four additional substitutions in Delta AY.4.2 (T95I, Y145H, A222V, and K458R). T95I is also present on the Omicron BA.1 spike and residue Y145 is also substituted in Omicron BA.1 (Y145D). A222V and K458R are unique to Delta AY.4.2. As the presence of these mutations in Delta AY.4.2 do not abrogate or enhance the aggregation of Delta AY.4.2 compared to Delta, they appear to have no effect on aggregation.

Most of the other mutations on the Delta and Delta AY.4.2 spikes are shared with other variants. The D614G mutation is present in all variants, substitution L452R located in the RBD is present in the Kappa variant (B.1.617.1), and a similar substitution L452Q occurs in the Lambda variant (C.37). A second RBD substitution at T478K is also found in Omicron BA.1 and BA.2. The mutation P681R is present in Kappa, and P681H exists in Alpha, Omicron BA.1, BA.2, and Mu (B.1.621). Finally, substitution mutation D950N is also shared with Mu. Because the non-Delta variants studied here do not aggregate, it is unlikely that any of their Delta-shared mutations can be aggregation-dominant.

There are, however, three residues, E156, F157, and R158, in the NTD of Delta and Delta AY.4.2 that are uniquely and identically mutated: substitution E156G, and deletions at F157 and R158. It is possible that these three mutations in the NTD are sufficient to bestow the aggregation property alone, or in the context of the other Delta mutations.

The clustering of Delta PVs could account for the faster and larger initial infection observed in entry assays with the Delta PVs [35]. Because the number of spike trimers

is larger on an aggregate comprising multiple PVs, and the cell surface contact area is larger for any collision between the aggregate and a target cell, the effective on-rate for aggregate binding should be larger, resulting in faster binding. Furthermore, the avidity of the aggregate to the target cell would be enhanced manyfold due to the multiple potential binding partners on a single contacting surface. Moreover, the increased dwell time at that contact area will allow for diffusional and conformational motions of proteins and lipids to increase the chance of membrane fusion, as these factors are important for avoiding hemifusion and promoting full fusion [56]. All these factors should lead to the relatively higher initial rate of PV entry into target cells from aggregated PVs. Whether or not aggregates could enable the simultaneous delivery of multiple copies of entry reporter genes to target cells is not clear, since the PVs need not display ACE2 and thus may not fuse to each other, even in an endosome; thus each virus in an aggregate may have to independently fuse to the endosomal membrane to place its genes to that cell's cytoplasm. Implicitly, there would be more overall binding events for unaggregated PVs, each at another site. However, if the probability for PV entry was low due to unbinding, then the factors discussed above to increase PV avidity would tend to increase overall fusion and its rate.

In summary, an ultrastructural analysis of retrovirus pseudotyped viral particles bearing SARS-CoV-2 spike variants led to a serendipitous discovery of significant aggregation when the Delta variant spike was expressed, but not upon expression of three other variant spikes. Viral aggregation can impart fitness benefits by protecting virions from environmental hazards and by effecting simultaneous delivery of multiple viral genomes, or collective infection [57,58]. Notably, collective infection can favor initial infection in some contexts [59]. Likely, the size and number of virions per aggregate is important to increasing infectivity—too large and it would effectively reduce infectious units below a threshold, too few virions in an aggregate and the benefit of collective infection is not gained. The unique property of the Delta spike to aggregate PVs may underlie the faster infection by Delta PVs. Furthermore, spike mediated aggregation could be part of the molecular mechanism by which Delta variant SARS-CoV-2 achieves increased transmissibility and faster infection with a higher viral load. The continued aggregation of PVs over time indicates that clustering may be mediated by interactions between spike tips, which in turn may indicate an adhesivity of the viral surface recognized by the immune system thus altering the balance of host antiviral response towards inflammation.

Author Contributions: Conceptualization, J.D.P. and J.Z.; methodology, J.D.P., W.F., D.M., F.Z. and P.S.B.; resources, J.L.; formal analysis, J.D.P., P.S.B., W.F., D.M. and F.Z.; investigation, J.D.P., J.L., W.F., D.M. and F.Z.; writing—original draft preparation, J.D.P. and J.Z.; writing—review and editing, J.D.P., J.Z., D.M., F.Z., P.S.B., W.F. and J.L.; visualization, J.D.P., D.M., F.Z. and P.S.B.; validation, J.D.P., J.Z. and P.S.B.; supervision, J.Z. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the intramural program of the NICHD (DIR).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data not published in the manuscript is available from the corresponding author.

Acknowledgments: The authors would like to thank the members of the SIB COV-2 VLP team including Elena Mekhedov, Hang Waters, and Glen Humphrey for their help in discussions. We also thank Rick Kuo-Jui Huang (from the National Cancer Institute) and Stéphane Mahé (from Thermo Fisher Scientific), for their technical support with the FEI Tecnai T20 electron microscope.

Conflicts of Interest: The authors declare no conflict of interest.

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