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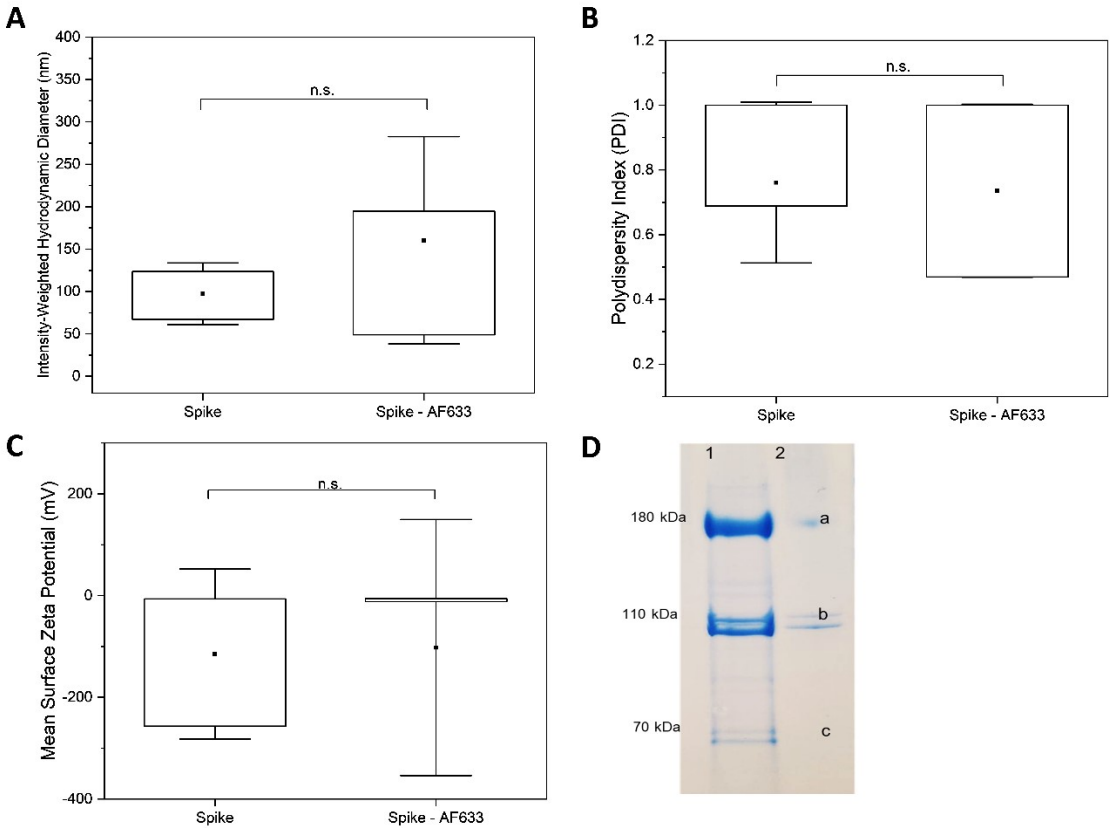
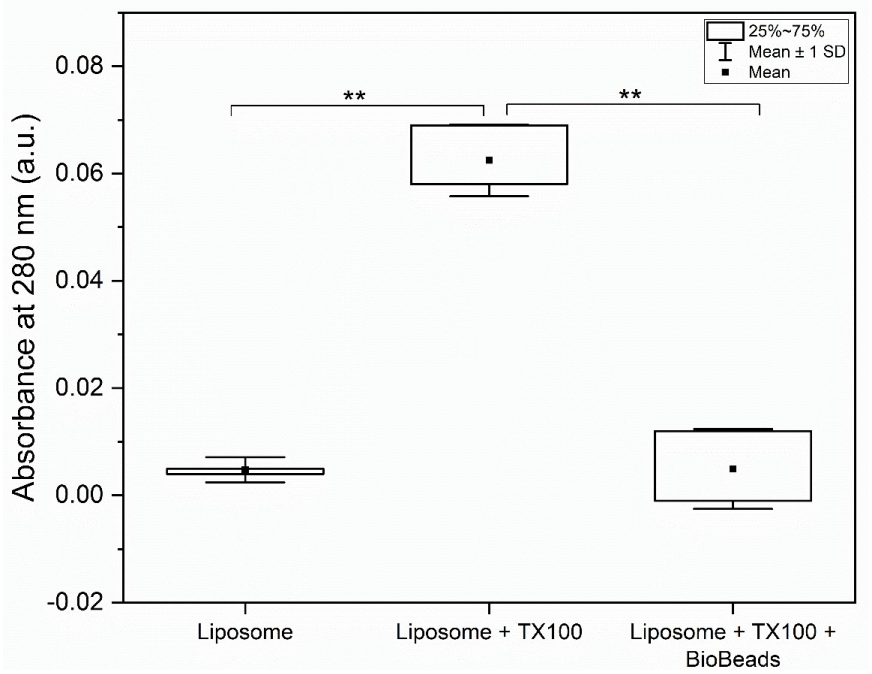
Electronic Supplementary Information

SARS-CoV-2 Virus-Like-Particles via Liposomal Reconstitution of Spike Glycoproteins

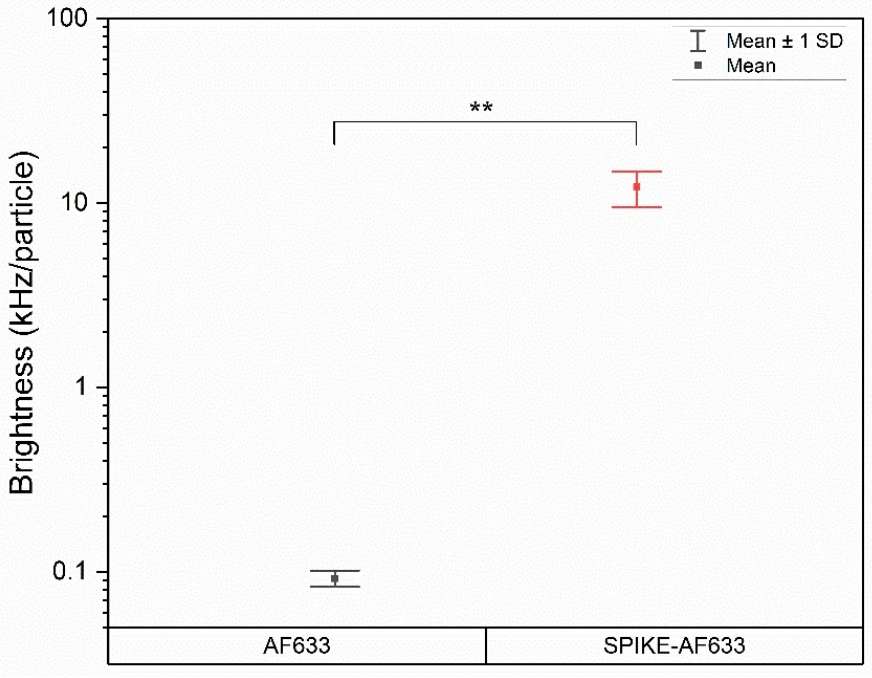
Sarah McColman*[ab], Klaidi Shkalla[ab], Pavleen Sidhu[ab] , Jady Liang[bc] , Selena Osman[ab], Norbert Kovacs[ab], Zainab Bokhari[ab] , Ana Carolina Forjaz Marques[abd] , Yuchong Li[bce] , Qiwen Lin[bce] , Haibo Zhang[bce] , and David T. Cramb*[abg]



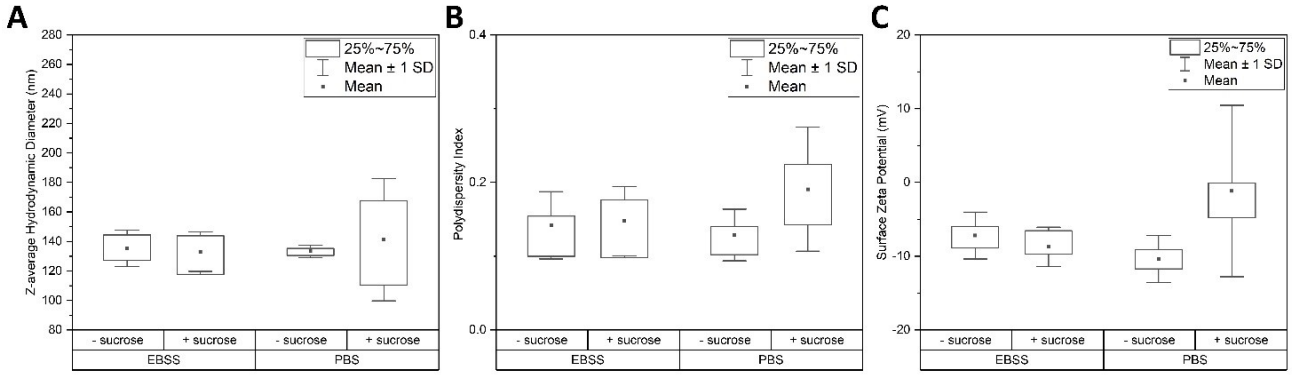
ESI Figure 1 *: Spike protein characterization pre- and post- labeling with Alexa Fluor™ 633 (AF633).* ***A)*** *Intensity-weighted hydrodynamic diameter values obtained from DLS analysis of spike protein solubilized in LMNG micelles as received from the supplier compared to the data for the same protein after labeling with AF633 and suspended in a solution of 5 μM LMNG with 2 mM sodium azide.* ***B)*** *Comparison of the polydispersity indices of LMNG-solubilized spike protein solutions before and after AF633 labeling.* ***C)*** *Comparison of the mean surface zeta potentials of LMNG-solubilized spike protein solutions before and after labeling with AF633.* ***D)*** *SDS-PAGE analysis of unlabeled (lane 1) and AF633-labeled (lane 2) spike proteins where (a) represents an intact monomer and (b) and (c) represent the S1 and S2 subunits of the spike protein, respectively. For panels A-C, data represents the mean (black square) of two independent liposome preparations, each measured in triplicate. Boxes show interquartile range and error bars designate ± standard deviation. Shapiro-Wilk normality tests were completed and because of non-normally distributed small populations, Kruskal-Wallis ANOVA tests were performed.*



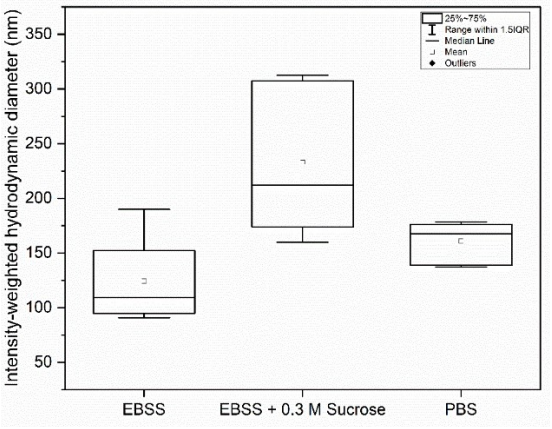
***ESI Figure 2**: Absorbance at 280 nm measured for Formulation 2 liposomes in EBSS before addition of 0.2 mM TX100, after the addition of 0.2 mM TX100, and after the 0.2 mM TX100 addition and subsequent detergent removal using Bio-Beads™ SM-2 resin. TX100 concentration for saturation of liposomes was selected to mimic reconstitution conditions. Bio-Beads™ were added in four increments of 3 mg each, over 20 hours. Absorbance measurements were conducted on a Nanodrop spectrophotometer with a path length of 0.1 cm. Data represents mean, interquartile range, and standard deviation of three independent preparations each measured in triplicate. Significance is shown as ** for p < 0.01 as calculated using Kruskal-Wallace ANOVA for non-normal distributions of data as determined by the Shapiro-Wilk normality test.*



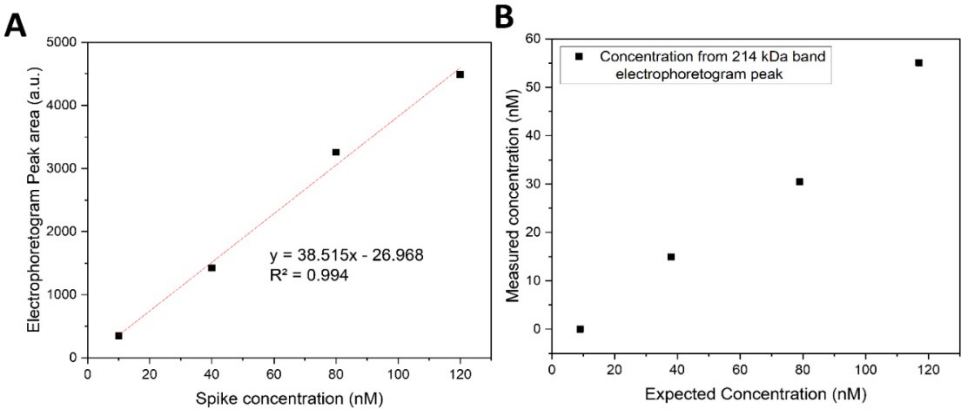
***ESI Figure 3**: Brightness per particle of unlabeled liposomes reconstituted with AF633 or Spike-AF633, measured using FCS. Data represents mean and standard deviation of three measurements of one independent reconstitution of each fluorophore. Significance is shown as ** (p < 0.01) as calculated by one-way ANOVA.*



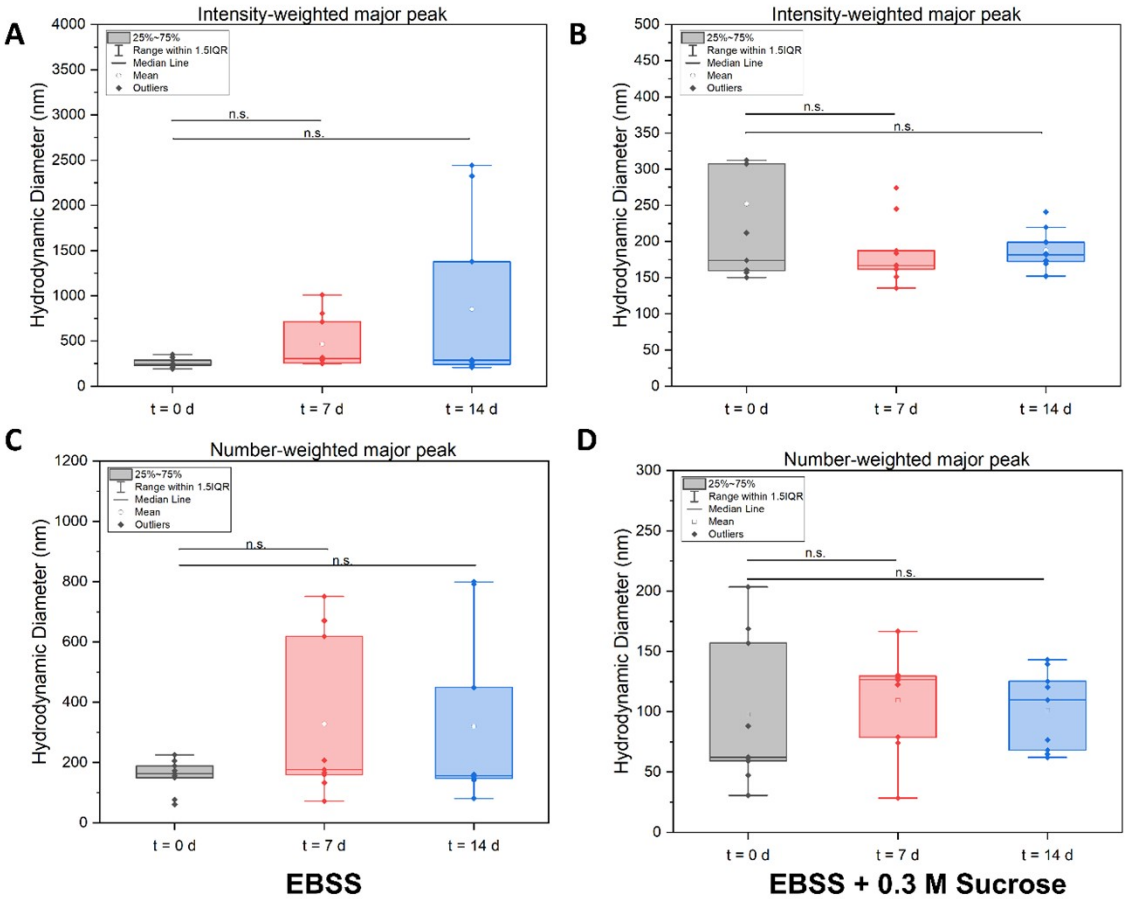
ESI Figure 4: (A) *Mean z-average hydrodynamic diameters of formulation 2 liposomes in four buffer systems using DLS.* ***(B)*** *Mean polydispersity indices of formulation 2 liposomes in four buffer systems using DLS.* ***(C)*** *Mean zeta (ζ) potential of formulation 2 liposomes in four buffer systems using ELS. Filled points represent mean of 3 independent samples (n=3 technical replicates for each). Bars represent ± standard deviation for these means. Two-way ANOVA was performed for all panels.*



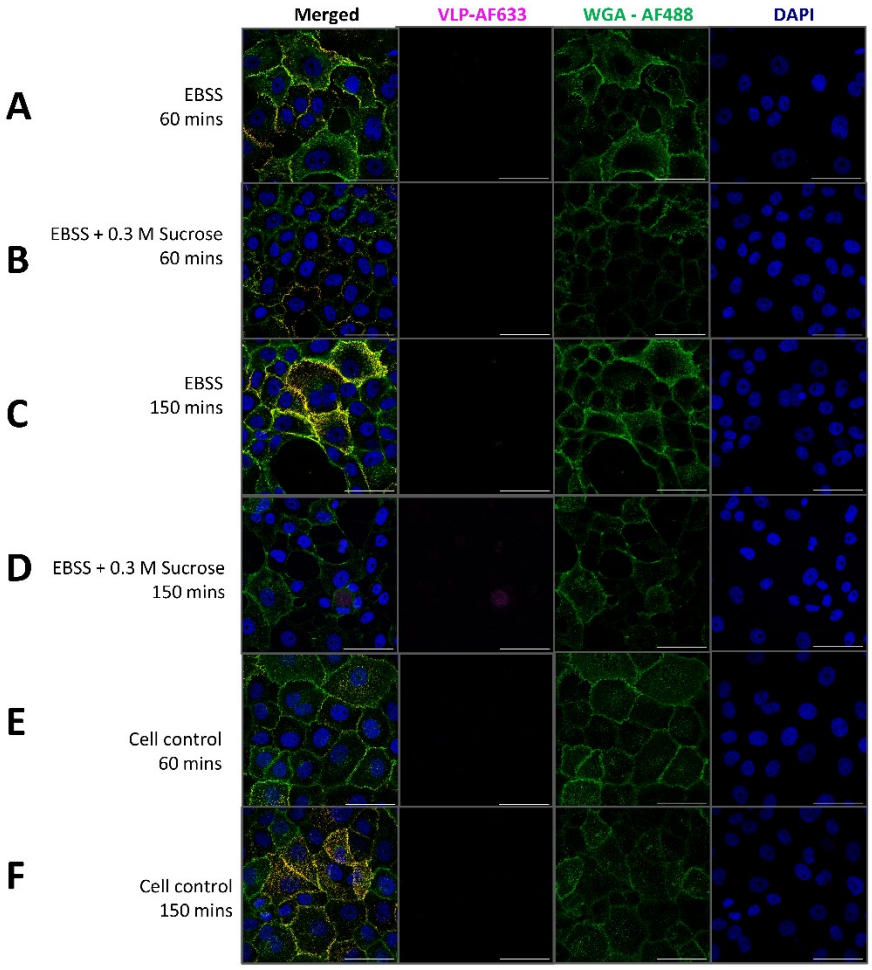
ESI Figure 5: *Intensity-weighted hydrodynamic diameter values obtained from DLS analysis of VLPs prepared with 50 nM spike protein in EBSS, EBSS + 0.3 M sucrose, or 1x PBS. Data represents three technical replicates from each of two independent biological replicates. Welch ANOVA was performed due to differences in variance determined by the Levene's test, and at a significance threshold of $p < 0.05$ no significant difference is seen between conditions.*



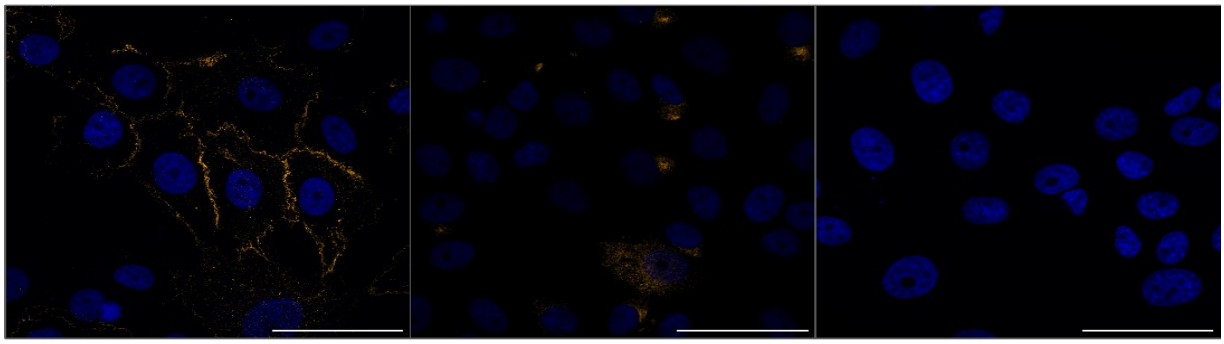
ESI Figure 6: A) *Standard curve of electrophoretogram peak areas versus spike protein concentration generated by analyzing the SDS-PAGE image in Main Figure 7 using FIJI.* ***B)*** *Concentration of spike in VLPs, plotted against the expected concentration as predicted by the initial amount of spike protein added to the reaction mixture. Concentrations were calculated from the electrophoretogram peak at apparent molecular weight of 214 kDa using the same SDS-PAGE in Main Figure 7.*



ESI Figure 7: A-B) *Intensity-weighted hydrodynamic diameter values obtained from DLS analysis of VLPs prepared with 50 nM spike protein in* ***A)*** *EBSS and* ***B)*** *EBSS + 0.3 M sucrose.* ***C-D)*** *Number-weighted hydrodynamic diameter values obtained from DLS analysis of VLPs prepared with 50 nM spike protein in* ***C)*** *EBSS and* ***D)*** *EBSS + 0.3 M sucrose. Measurements were taken on day 0, 7, and 14 since preparation, and between timepoints VLP samples were stored at 4°C. Statistical analysis was performed using one-way ANOVA when the Shapiro-Wilk and Levene's tests indicated data normality and homogeneity of variance, respectively. Welch's ANOVA was used when data normality and variance criteria were not met. Data represents three technical replicates each for three independent formulations of VLPs in each buffer system.*

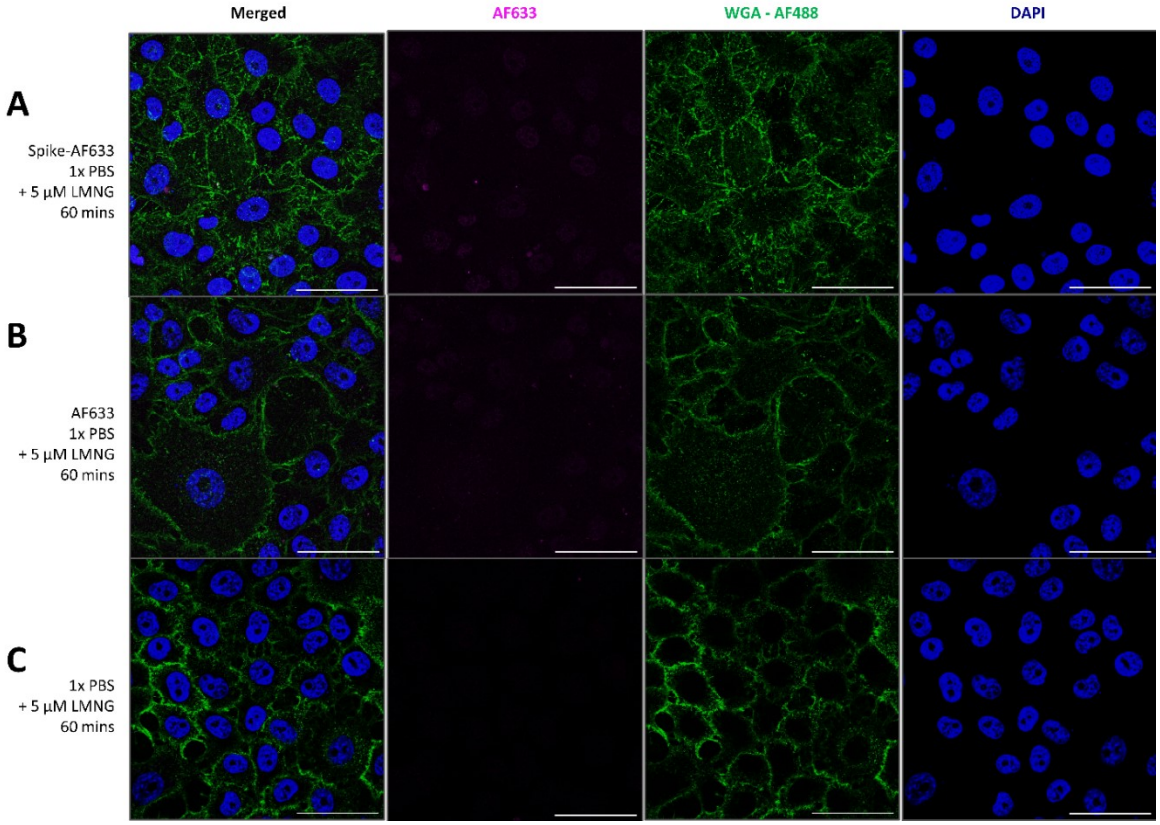


ESI Figure 8: *Representative confocal fluorescence microscopy images of Calu-3 cells treated for 60 minutes with* ***A)*** *EBSS and* ***B)*** *EBSS + 0.3 M sucrose, and for 150 minutes with* ***C)*** *EBSS and* ***D)*** *EBSS + 0.3 M sucrose. Cell controls treated with MEM-α media for* ***E)*** *60 or* ***F)*** *150 minutes are incuded to compare cell morphology with those treated with the two buffer systems. Cell membranes are labelled with WGA-AF488 (green) and nuclear staining via DAPI is shown in blue. Scale bars represent 50 µm.*

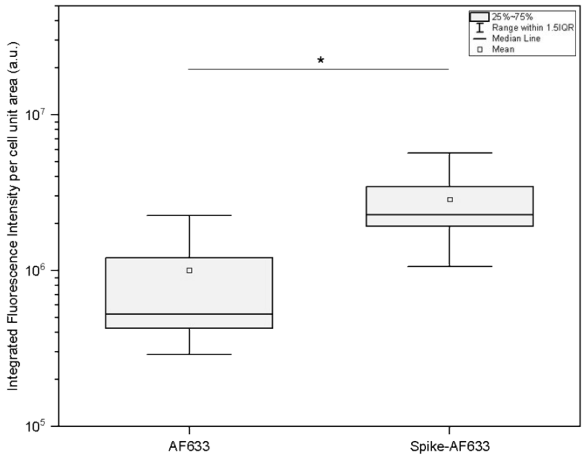


IgG	-	+	-
1° Ab	+	-	+
2° Ab	+	-	-

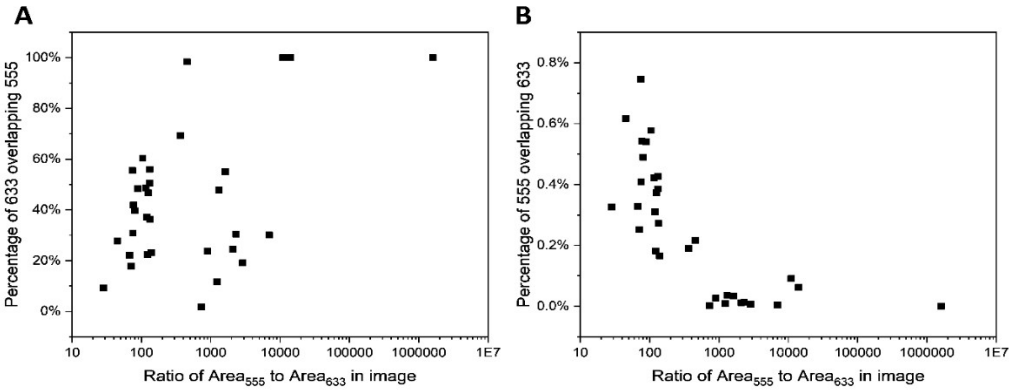
ESI Figure 9: *Representative confocal fluorescence microscopy images of Calu-3 cells treated for 60 minutes with immunofluorescence control solutions, showing membrane-localized ACE-2 fluorescence (orange) only in those cells treated with both primary and secondary antibodies. Nuclear staining via DAPI is shown in blue. Scale bars represent 50 µm.*



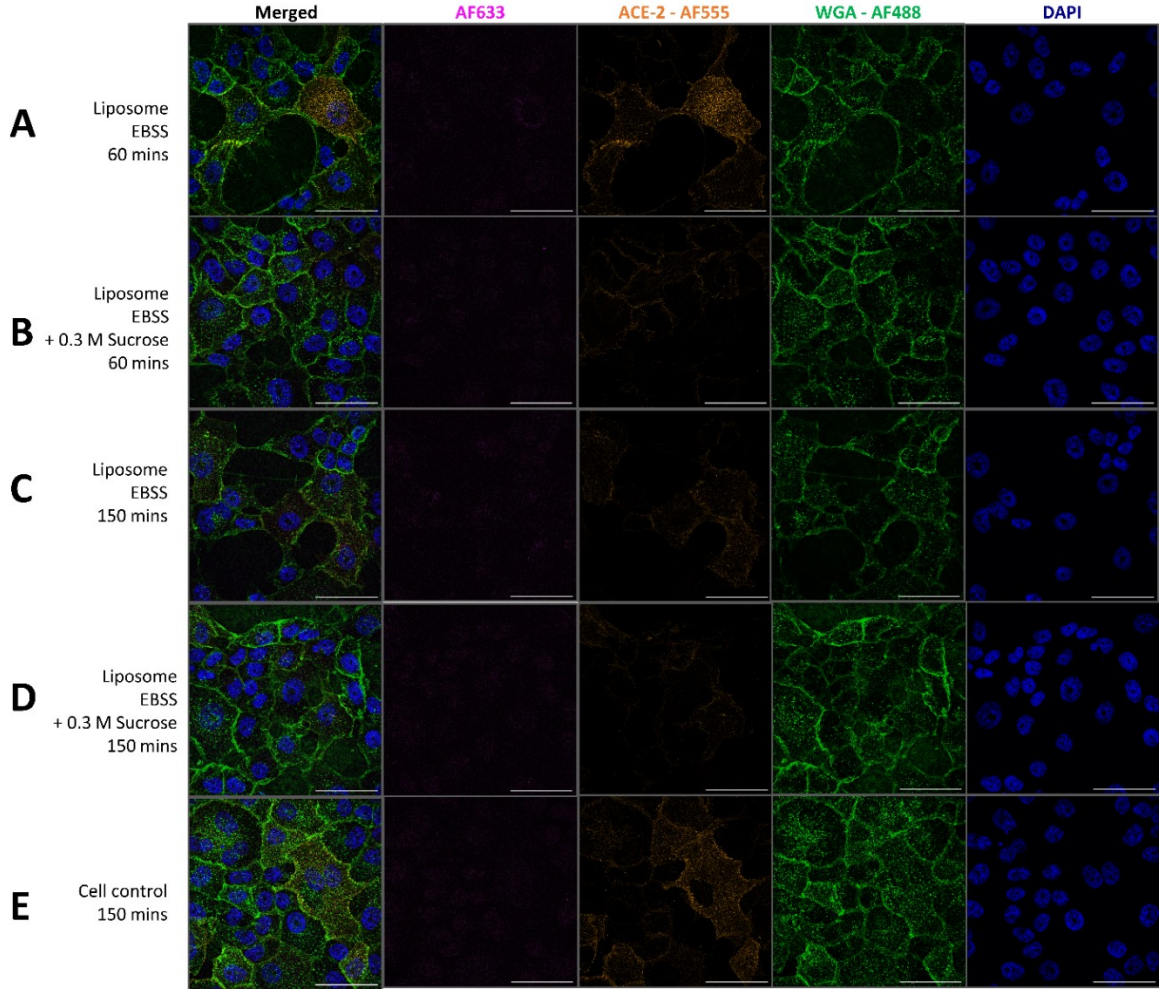
***ESI Figure 10**: Confocal fluorescence microscopy images of Calu-3 cells treated for 60 minutes with **A)** 25 nM Spike-AF633 in 1x PBS with 5 uM LMNG and 2 mM sodium azide, **B)** 850 nM AF633 in 1x PBS with 5 uM LMNG and 2 mM sodium azide, and **C)** 1x PBS with 5 uM LMNG and 2 mM sodium azide. Spike proteins used in this experiment were labeled so that each spike protein contained 34 dye molecules as calculated during the labeling reaction. AF633 is shown by pink signal, cell membranes are labelled with WGA-AF488 (green) and nuclear staining via DAPI is shown in blue. Scale bars represent 50 µm.*



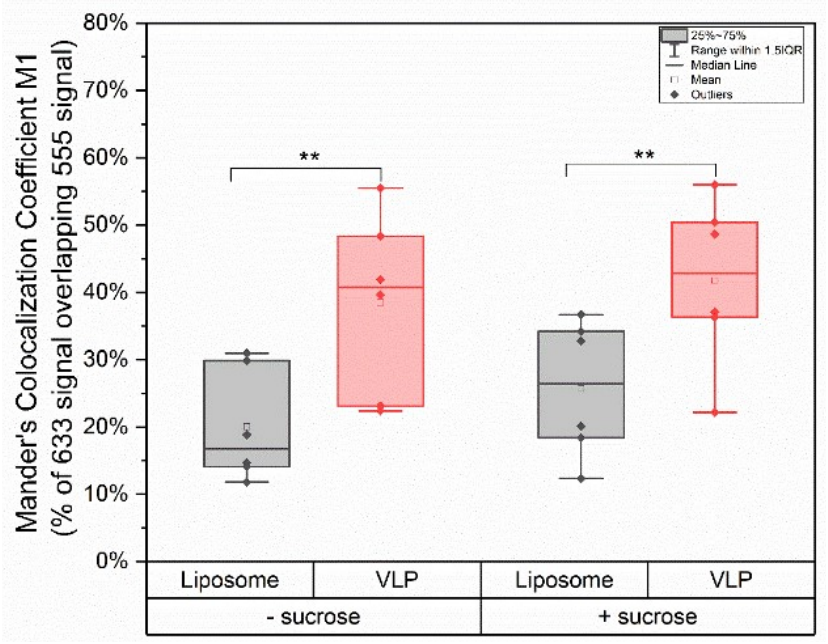
***ESI Figure 11:** Integrated fluorescence intensity per cell unit area compared between CALU-3 cells treated with AF633 and equivalent cells treated with Spike-AF633. AF633 treatment consisted of 850 nM AF633 in 5 uM LMNG and 2 mM sodium azide, and the spike treatment consisted of 25 nM Spike-AF633 in the same buffer with each spike protein containing 34 dye molecules as calculated during the labeling reaction. Data represents mean, median, and interquartile range of integrated fluorescence intensity of 160 cells analyzed for each treatment, retrieved from three independent images each of three independent biological replicates. Significance is shown as ** $p < 0.01$, as calculated using Kruskal-Wallace ANOVA for non-normal distributions of data as determined by the Shapiro-Wilk normality test.*



ESI Figure 12: (A) *M1 colocalization coefficient between AF633 and AF555 (percent of AF633-positive pixels that also are positive for AF555) in CALU-3 cells treated with VLPs plotted as the function of the ratio of 555-positive to 633-positive pixels in the respective image.* ***(B)*** *M2 colocalization coefficient between AF633 and AF555 (percent of AF555-positive pixels that also are positive for AF633) in CALU-3 cells treated with VLPs plotted as the function of the ratio of 555-positive to 633-positive pixels in the respective image.*



ESI Figure 13: *Confocal fluorescence microscopy images of Calu-3 cells treated for* ***A)*** *60 minutes with formulation 2 liposomes in EBSS,* ***B)*** *60 minutes with formulation 2 liposomes in EBSS + 0.3 M sucrose,* ***C)*** *150 minutes with formulation 2 liposomes in EBSS,* ***D)*** *150 minutes with formulation 2 liposomes in EBSS + 0.3 M sucrose, and* ***E)*** *150 minutes with MEM-α cell media. Staining shows AF555-labeled ACE-2 (orange), cell membranes with WGA-AF488 (green) and nuclear staining via DAPI is shown in blue. Scale bars represent 50 µm.*



ESI Figure 14: *M1 colocalization coefficients between AF633 and AF555 (percent of AF633-positive pixels that also are positive for AF555) in CALU-3 cells treated for 60 minutes with Liposomes or VLPs in EBSS (- sucrose) or EBSS + 0.3 M sucrose (+ sucrose). Data represents mean, median, and interquartile range of M1 values, retrieved from three independent images each of two independent biological replicates. Significance is shown as ** $p < 0.01$ from Tukey's test comparing means within 2-way ANOVA.*

ESI Table 1: *Composition of all liposome formulations prepared and tested, catalogued by formulation code and showing mole percent of each membrane component.*

	Proportion of membrane component (mol %)							
ID	**DOPC**	**DPPC**	**DOPE**	**DPPE**	**DOPI**	**DPPI**	**Cholesterol**	**PEG-1000**
F1	89			10				
F2	79			20				
F3	74			25				
F4	89		10					
F5	79		20					
F6	74		25					
F7		89		10				
F8		79		20				
F9		74		25				
F10		89	10					
F11		79	20					
F12		74	25					
F13	85			10	5			
F14	80			9	10			
F15	75			19	5			
F16	71			18	10			
F17	85			10		5		
F18	80			9		10		
F19	76			9		15		
F20	75			19		5		
F21	71			18		10		
F22	67			17		15		
F23	70			24		5		
F24	67			23		10		
F25	63			21		15		
F26	85		10			5		
F27	80		9			10		
F28	76		9			15		
F29	75		19			5		
F30	71		18			10		
F31	67		17			15		
F31	70		24			5		
F32	67		23			10		
F33	63		21			15		
F34		85		10		5		
F35		80		9		10		
F36		76		9		15		
F37		75		19		5		
F38		71		18		10		
F39		67		17		15		
F40		70		24		5		
F41		67		23		10		
F42		63		21		15		
F43		85	10			5		
F44		75	19			5		
F45		67	17			15		
F46		70	24			5		
F47		67	23			10		
F48		63	21			15		

	Proportion of membrane component (mol %)							
ID	**DOPC**	**DPPC**	**DOPE**	**DPPE**	**DOPI**	**DPPI**	**Cholesterol**	**PEG-1000**
F49	82			9			9	
F50	75			8			17	
F51	69			8			23	
F52	72			18			9	
F53	66			17			17	
F54	61			16			23	
F55	68			23			9	
F56	62			21			17	
F57	57			19			23	
F58	82		9				9	
F59	75		8				17	
F60	69		8				23	
F61	72		18				9	
F62	66		17				17	
F63	61		16				23	
F64	68		23				9	
F65	62		21				17	
F66	57		19				23	
F67		82		9			9	
F68		75		8			17	
F69		69		8			23	
F70		72		18			9	
F71		66		17			17	
F72		61		16			23	
F73		68		23			9	
F74		62		21			17	
F75		57		19			23	
F76		82	9				9	
F77		75	8				17	
F78		69	8				23	
F79		72	18				9	
F80		61	16				23	
F81		68	23				9	
F82		62	21				17	
F83		57	19				23	
F84	78			9	5		9	
F85	66			7	4		23	
F86	80			9	10			
F87		99						
F88		99						
F89	74			25				
F90	74			25				
F91	73			8			16	2
1		66	17				17	
2	78			9	5		9	
3	71			8	4		17	
2^{PEG}	76			9	4		9	2
3^{PEG}	70			8	4		16	2
1^{PEG}	65			16			16	2

ESI Table 2: *Student's t-test t statistic, degrees of freedom (DF), and p values for two-sample t-tests reported in Figure 4A-D.*

Figure	Data comparison	t Statistic	DF	p Value
Figure 4A	Formulation 1 Day 0 / Day 4 hydrodynamic diameter (1x PBS)	-2.8	28	8.8E-3
	Formulation 2 Day 0 / Day 4 hydrodynamic diameter (1x PBS)	-1.7	26	9.7E-2
	Formulation 3 Day 0 / Day 4 hydrodynamic diameter (1x PBS)	3.3	25	2.8E-3
	Formulation 1PEG Day 0 / Day 4 hydrodynamic diameter (1x PBS)	-3.6	27	1.2E-3
	Formulation 2PEG Day 0 / Day 4 hydrodynamic diameter (1x PBS)	-3.9	23	6.5E-4
	Formulation 3PEG Day 0 / Day 4 hydrodynamic diameter (1x PBS)	-0.15	18	8.8E-1
Figure 4B	Formulation 1 Day 0 / Day 4 concentration (1x PBS)	0.45	28	6.5E-1
	Formulation 2 Day 0 / Day 4 concentration (1x PBS)	0.96	26	3.4E-1
	Formulation 3 Day 0 / Day 4 concentration (1x PBS)	-2.14	25	4.2E-2
	Formulation 1PEG Day 0 / Day 4 concentration (1x PBS)	-0.23	27	8.2E-1
	Formulation 2PEG Day 0 / Day 4 concentration (1x PBS)	2.71	23	1.2E-2
	Formulation 3PEG Day 0 / Day 4 concentration (1x PBS)	-0.53	18	6.0E-1
Figure 4C	Formulation 1 Day 0 / Day 4 hydrodynamic diameter (1x PBS + 0.3 M sucrose)	-3.20	28	3.4E-3
	Formulation 2 Day 0 / Day 4 hydrodynamic diameter (1x PBS + 0.3 M sucrose)	-1.16	28	2.6E-1
	Formulation 3 Day 0 / Day 4 hydrodynamic diameter (1x PBS + 0.3 M sucrose)	0.32	23	7.5E-1
	Formulation 1PEG Day 0 / Day 4 hydrodynamic diameter (1x PBS + 0.3 M sucrose)	-1.91	29	6.5E-2
	Formulation 2PEG Day 0 / Day 4 hydrodynamic diameter (1x PBS + 0.3 M sucrose)	-2.04	28	5.1E-2
	Formulation 3PEG Day 0 / Day 4 hydrodynamic diameter (1x PBS + 0.3 M sucrose)	-2.56	27	1.6E-2
Figure 4D	Formulation 1 Day 0 / Day 4 concentration (1x PBS + 0.3 M sucrose)	5.69	28	4.3E-6
	Formulation 2 Day 0 / Day 4 concentration (1x PBS + 0.3 M sucrose)	2.13	28	4.2E-2
	Formulation 3 Day 0 / Day 4 concentration (1x PBS + 0.3 M sucrose)	-3.34	24	2.7E-3
	Formulation 1PEG Day 0 / Day 4 concentration (1x PBS + 0.3 M sucrose)	7.73	29	1.6E-8
	Formulation 2PEG Day 0 / Day 4 concentration (1x PBS + 0.3 M sucrose)	-1.01	28	3.2E-1
	Formulation 3PEG Day 0 / Day 4 concentration (1x PBS + 0.3 M sucrose)	1.32	27	2.0E-1

ESI Table 3*: F statistic, degrees of freedom between groups (DF_1), and degrees of freedom within groups (DF_2), and p values for one-way ANOVA statistical analyses.*

Figure	Data comparison	F	DF_1	DF_2	p value
Figure 6B	Diffusion coefficient Spike-AF633 vs VLP-AF633	236.54	1	47	5.7E-20
Figure 6C	Brightness per particle Spike-AF633 vs VLP-AF633	15.63	2	9	1.2E-3
Figure 10A	Mander's colocalization Liposome vs VLP EBSS	8.21	1	10	1.7E-2
Figure 10B	Mander's colocalization Liposome vs VLP EBSS + 0.3 M sucrose	6.09	1	10	3.3E-2
ESI Figure 3	AF633 / Spike-AF633 (brightness)	62.28	1	4	1.4E-3
ESI Figure 7	Day 0 / Day 7 / Day 14 (Number-weighted) for VLPs in EBSS + 0.3 M Sucrose	0.15	2	24	8.6E-2

ESI Table 4 *: Chi-Square value, degrees of freedom (DF), and p values for Kruskal-Wallis ANOVA statistical analyses.*

Figure	Data comparison	Chi-Square	DF	p value
ESI Figure 1A	Spike / Spike-AF633 (diameter)	8	8	0.43
ESI Figure 1B	Spike / Spike-AF633 (PDI)	5	4	0.29
ESI Figure 1C	Spike / Spike-AF633 (Zeta Potential)	0.48	1	0.49
ESI Figure 2	Liposome / Liposome + TX100 / Liposome + TX100 + BioBeads	18.61	2	9.1E-5
	Liposome + TX100 / Liposome + TX100 + BioBeads	0.93	1	0.34
ESI Figure 5	EBSS/EBSS-Sucrose/PBS (VLP Diameter)	4.52	2	0.054
ESI Figure 11	AF633 vs Spike-AF633 Integrated Fluorescence Intensity	159.60	1	1.4E-36

ESI Table 5: *Total degrees of freedom (DF), ANOVA factors with corresponding DF within that factor, F statistic, and p values for two-way ANOVA statistical analyses.*

Figure	Data Comparison	DF_{total}	Factor	DF_{factor}	F	p Value
Figure 5A	6 Formulations Zeta Potential 1x PBS / 1x PBS + 0.3 M sucrose	35	formulation	5	1.05	4.1E-1
			buffer	1	2.34	1.4E-1
			interaction	5	2.96	3.2E-2
Figure 5B	6 Formulations hydrodynamic diameter 1x PBS / 1x PBS + 0.3 M sucrose	35	formulation	5	1.89	1.3E-1
			buffer	1	3.36	7.9E-2
			interaction	5	0.59	7.0E-1
Figure 5C	6 Formulations PDI 1x PBS / 1x PBS + 0.3 M sucrose	35	formulation	5	0.88	5.1E-1
			buffer	1	0.20	6.6E-1
			interaction	5	0.93	4.8E-1
ESI Figure 4A	Liposome hydrodynamic diameter EBSS/PBS and +/- sucrose	31	buffer	1	0.10	7.6E-1
			sucrose	1	0.09	7.7E-1
ESI Figure 4B	Liposome PDI EBSS/PBS and +/- sucrose	32	buffer	1	0.46	5.0E-1
			sucrose	1	2.31	1.4E-1
ESI Figure 4C	Liposome Zeta Potential EBSS/PBS and +/- sucrose	28	buffer	1	0.70	4.1E-1
			sucrose	1	2.08	1.6E-1
ESI Figure 7	Mander's colocalization Liposome vs VLP EBSS + 0.3 M sucrose	23	particle	1	14.21	1.2E-3
			sucrose	1	0.97	3.4E-1

ESI Table 6*: F value, degrees of freedom (DF), and p values for Welch's ANOVA statistical analyses.*

Figure	Data comparison	F	DF	p value
ESI Figure 7A	Day 0 / Day 7 / Day 14 (Intensity-weighted) for VLPs in EBSS	3.74	2	5.7E-2
ESI Figure 7B	Day 0 / Day 7 / Day 14 (Intensity-weighted) for VLPs in EBSS + 0.3 M Sucrose	0.72	2	5.0E-1
ESI Figure 7C	Day 0 / Day 7 / Day 14 (Number-weighted) for VLPs in EBSS	2.92	2	9.5E-2