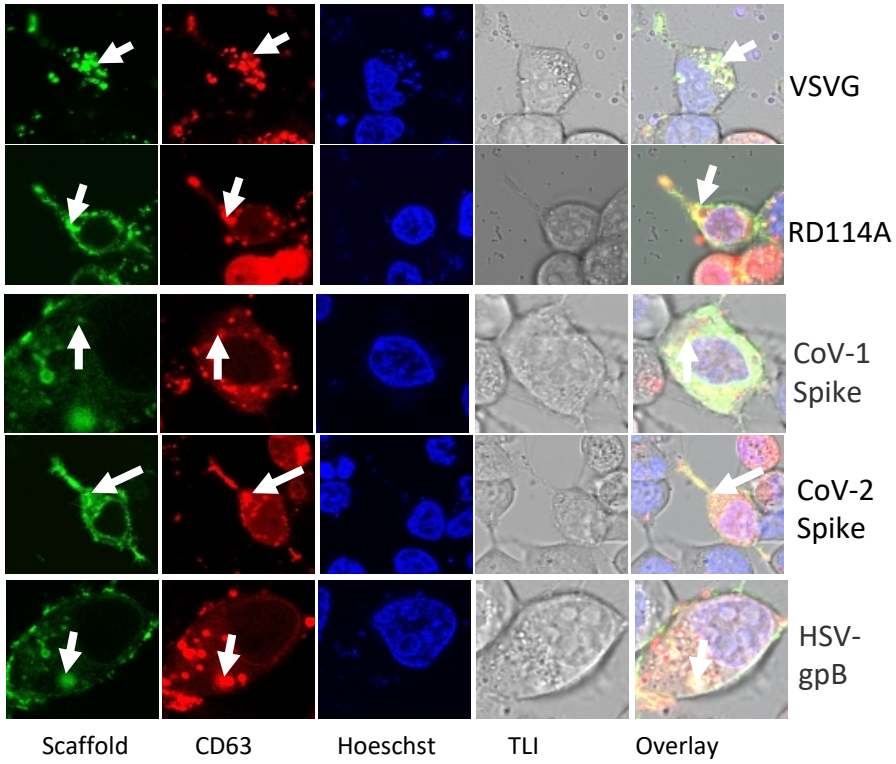
Figure S1

A Co-localization of scaffolds with CD63

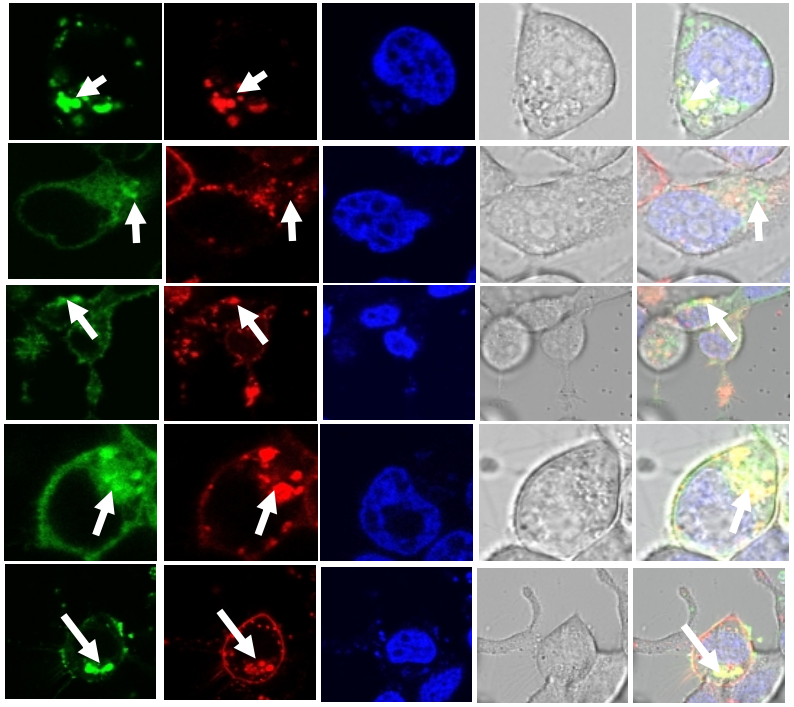


B Co-efficiency of co-efficiency

Scaffolds	CD63			
	24 hr	48 hr	72 hr	Mean ± SD
VSVG	0.884	0.809	0.927	0.873 ± 0.06
RD114A	0.949	0.595	0.739	0.761 ± 0.18
CoV-1 Spike	0.823	0.832	0.882	0.846 ± 0.03
CoV-2 Spike	0.925	0.774	0.688	0.796 ± 0.12
HSV-B	0.899	0.81	0.844	0.851 ± 0.04

Figure S1. Co-localization of viral GP scaffolds with the exosome marker CD63.
(**A**) Representative fluorescence and transmitted light images (TLI) of 293T cells co-transfected with viral envelope GP-GFP fusion constructs and CD63-RFP. Following transfection, images were recorded for three consecutive days and representative images at 72 hours (hr) with Hoeschst stain are shown. Fluorescence signals reveal co-localization of GP-GFP scaffolds (green) with CD63-RFP (red), indicating their association with exosomal or multivesicular body (MVB) structures. Arrows indicate endosomal, exosomal, or MVB structures. Scale bars: 10 μm. Arrows indicate the co-localization of GP scaffolds and CD63.
(**B**) Co-localization efficiencies for different scaffolds were analyzed using ImageJ and are presented as mean ± SD using images recorded at 24, 48, and 72 hr following transfection.

A Co-localization of scaffolds with XPACK-RFP



B Co-efficiency of colocalization

Scaffolds	XPACK-RFP			
	24 hr	48 hr	72 hr	Mean ± SD
VSVG	0.479	0.856	0.922	0.752 ± 0.24
RD114A	0.897	0.799	0.777	0.824 ± 0.06
CoV-1 Spike	0.745	0.824	0.718	0.762 ± 0.06
CoV-2 Spike	0.806	0.845	0.944	0.865 ± 0.07
HSV-B	0.803	0.722	0.659	0.728 ± 0.07

Figure S2. Co-localization of GP-GFP scaffolds with the exosome marker XPACK.
(**A**) Representative fluorescence and transmitted light images (TLI) of 293T cells co-transfected with viral envelope GP-GFP fusion constructs and XPACK-RFP. Images were recorded for three consecutive days post-transfection. Representative images at 72 hours (hr) are shown. Fluorescence signals reveal co-localization of GP-GFP scaffolds (green) with XPACK-RFP (red), indicating their association with exosomal or multivesicular body (MVB) structures. Arrows indicate endosomal, exosomal, or MVB structures. Scale bars: 10 μm.
(**B**) Co-localization efficiencies of GP scaffolds with XPACK were analyzed using ImageJ. The results are presented as mean ± SD of images recorded at three different time points (24, 48, and 72 hr).

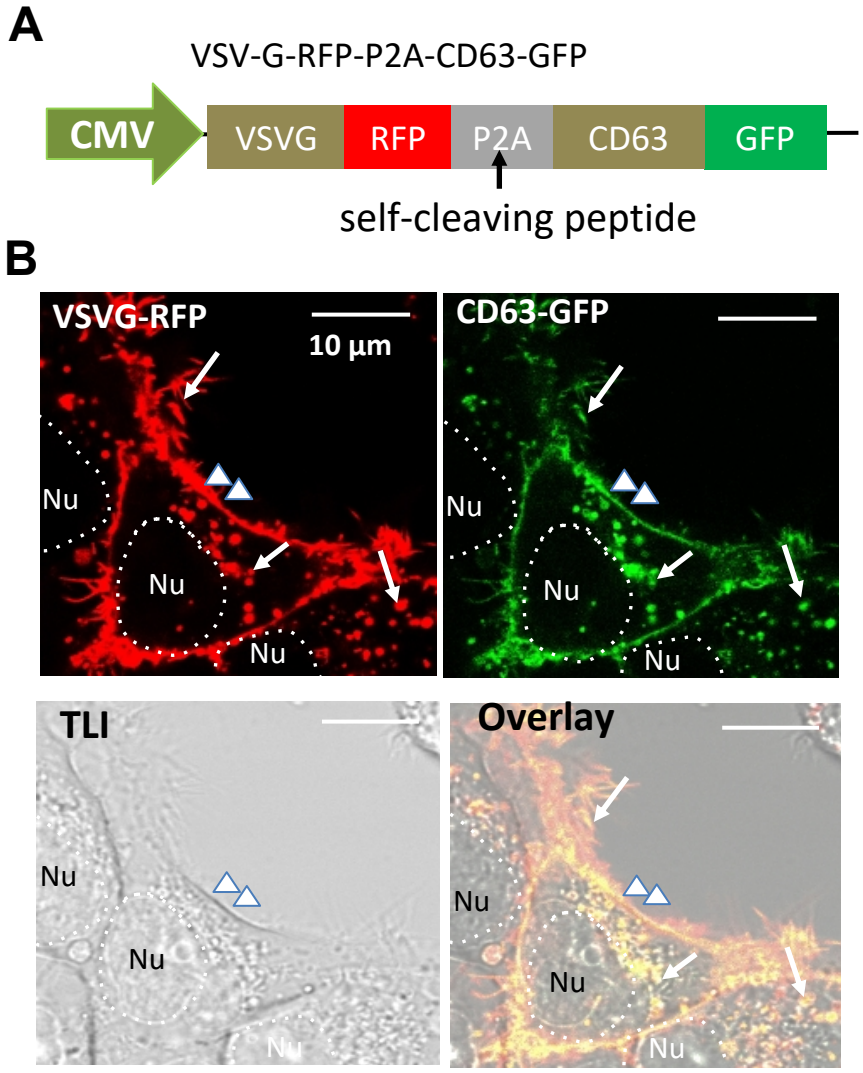
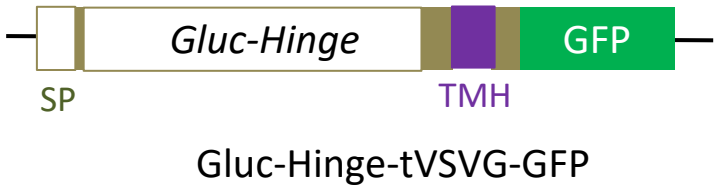


Figure S3. Co-localization of VSVG-RFP with the exosome marker CD63-GFP using a bicistronic expression cassette

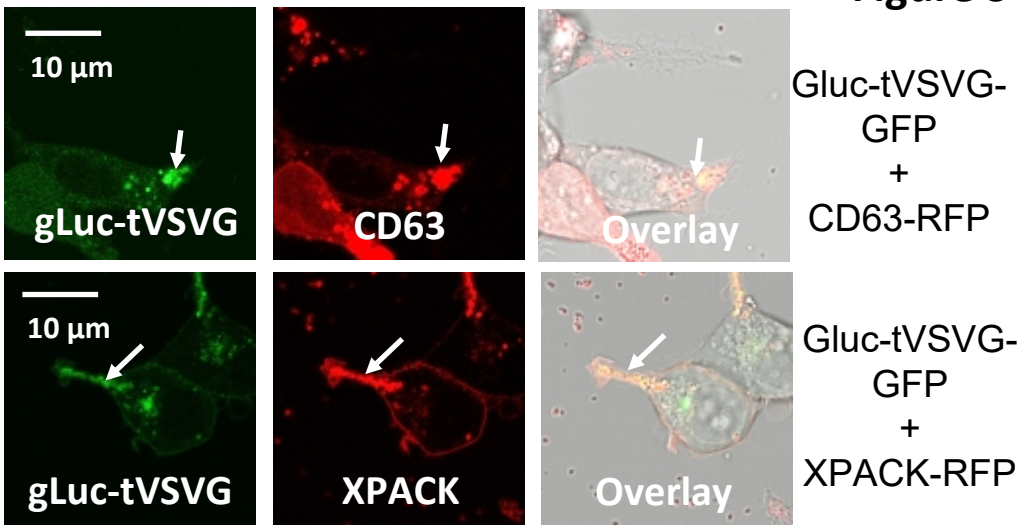
(**A**) Schematic representation of the chimeric fusion construct encoding VSVG-RFP and CD63-GFP, linked by a self-cleaving peptide (P2A). (**B**) Co-localization analysis of VSVG-RFP with CD63-GFP. Fluorescence and transmitted light images (TLI) of 293T cells were recorded 48 hours post-transfection. VSVG-RFP (red) and CD63-GFP (green) fluorescence signals show strong co-localization, indicating their association with exosomal or multivesicular body (MVB) structures. ImageJ analysis quantified a co-localization coefficient of 0.876, supporting a robust association. Arrows highlight endosomal, exosomal, or MVB structures. Dashed lines indicate the nucleus (Nu). Scale bars: 10 µm.

Figure S4

A



B



C

Constructs	Co-Efficiency of Co-localization			
	24 hr	48 hr	72 hr	Mean ± SD
Gluc-tVSVG-GFP + CD63-RFP	0.818	0.612	0.816	0.911 ± 0.002
Gluc-tVSVG-GFP + XPACK-RFP	0.883	0. 791	0.571	0.722 ± 0.127

Figure S4. Co-localization of truncated VSVG (tVSVG)–tagged with gLuc-Hinge-GFP and exosomal markers CD63 and XPACK. (**A**) Schematic of the Gluc-Hinge–tVSVG–GFP construct. The reporter includes a Gaussia luciferase (gLuc) and CD8 hinge domain fused to the transmembrane helix (TMH) and cytoplasmic tail of VSVG, followed by GFP. (**B**) Confocal microscopy of 293T cells transfected with gLuc-Hinge-tVSVG-GFP, either alone or in combination with exosomal markers CD63-RFP (top) or XPACK-RFP (bottom). Gluc-tVSVG-GFP localized to intracellular puncta (arrows), and merged images show strong co-localization with CD63 or XPACK. Transmitted light image (TLI) and overlays are shown. Scale bars, 10 µm. (**C**) Quantitative analysis of co-localization efficiency at 24, 48, and 72 hours post-transfection. gLuc-tVSVG-GFP co-localized with CD63-RFP (mean r = 0.911 ± 0.002**)** and XPACK-RFP (mean r = 0.722 ± 0.127). Data represent mean ± SD from three time points (n=3).

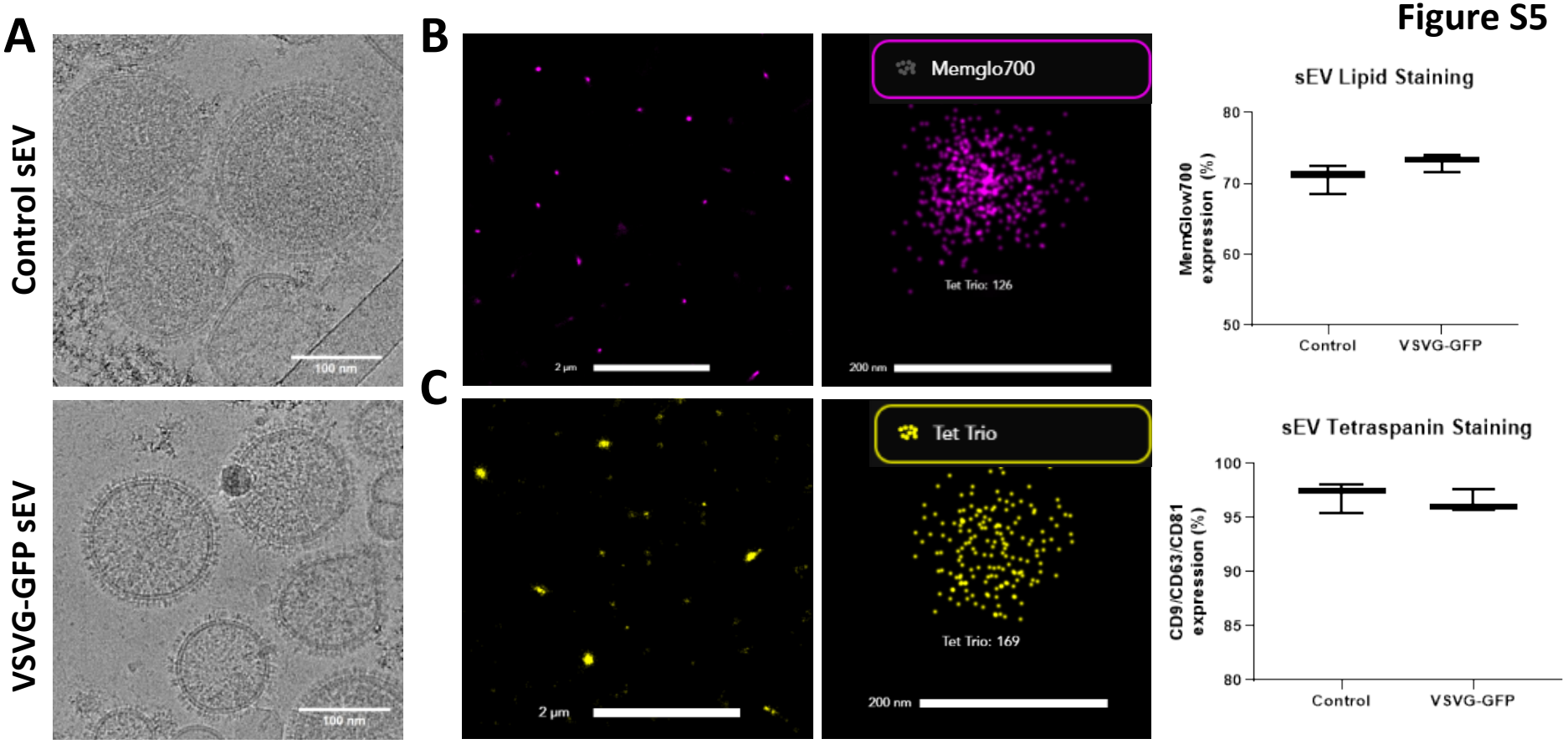


Figure S5. Biophysical, Chemical and Immunologic characterization of isolated sEVs. (**A**) Representative cryogenic electron microscopy images of control sEVs and VSVG-GFP sEVs, taken at 45,000x magnification. Scale bars represent 100 nm. (**B**) Super-resolution microscopy visualization of control sEVs labeled with lipophilic membrane dye MemGlow700. Lipid-stained sEV clusters are shown in wide view with a 2 µm scale bar and at the single EV scale with a 200 nm scale bar. Quantification of MemGlow700+ sEV clusters are shown in the box and whisker plot on the right. Data represent mean ± SD from three fields of view (n=3). (**C**) Control sEVs stained for CD9, CD63, and/or CD81 tetraspanins with pan-tetrapanin antibodies. Quantification of MemGlow700+ sEV clusters are shown in the box and whisker plot on the right. Data represent mean ± SD from three fields of view (n=3).

Table S1. Co-efficiency of co-localization of ectodomain-deleted viral envelope glycoproteins with the exosome markers of CD63 and XPACK

Constructs	Co-Efficiency of Colocalization			
	24 hr	48 hr	72 hr	Mean ± SD
HSV-dgpB-GFP + CD63-RFP	0.913	0.911	0.909	0.911 ± 0.002
HSV-dgpB- + XPACK-RFP	0.644	0.869	0.653	0.722 ± 0.127
CoV-1 dSpike-GFP + CD63-RFP	0.756	0.732	0.631	0.706 ± 0.066
CoV-1 dSpike-GFP + XPACK-RFP	0.573	0.537	0.356	0.489 ± 0.116

Note: Co-localization co-efficiency of viral envelope glycoproteins (HSV-dgpB or CoV-1 dSpike) tagged with GFP and exosomal markers (CD63 or XPACK) tagged with RFP at 24, 48, and 72 hours (hr). Values represent mean ± SD from three time points (n = 3).