

Supplementary Information for

Dynamic structures of dengue virus serotype 2 secreted NS1 and their interactions with heparan sulfate

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The supplementary information includes:

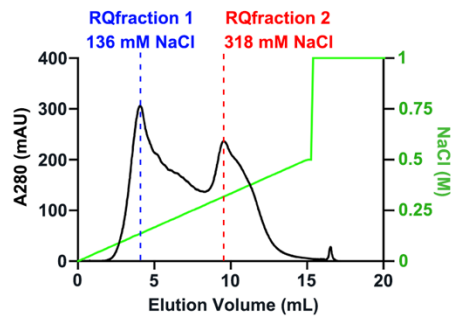
Supplementary Figure 1 to 13

Supplementary Table 1 to 2

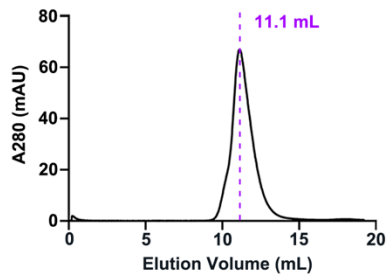
Supplementary Methods

Supplementary References

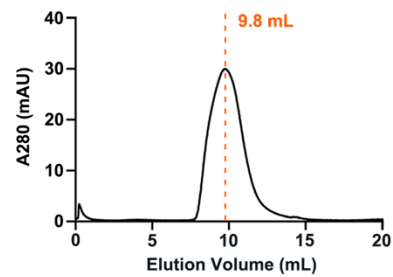
a Resource Q chromatography



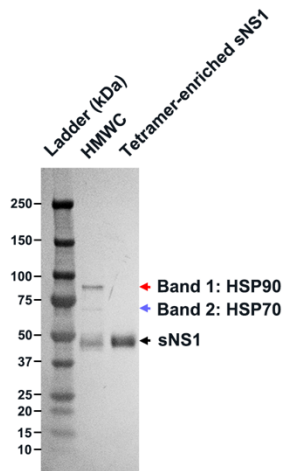
b Gel filtration chromatography of RQfraction 1 Tetramer-enriched sNS1 fraction



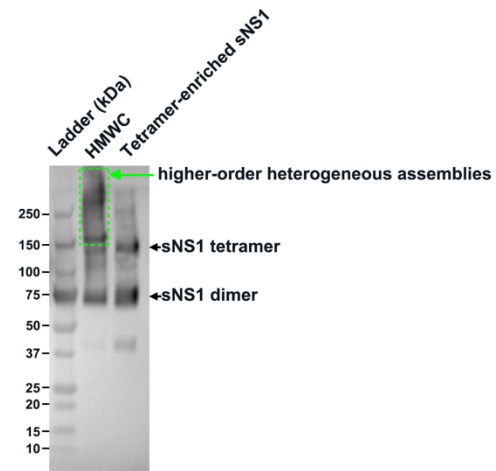
c Gel filtration chromatography of RQfraction 2 High molecular weight complex (HMWC) fraction



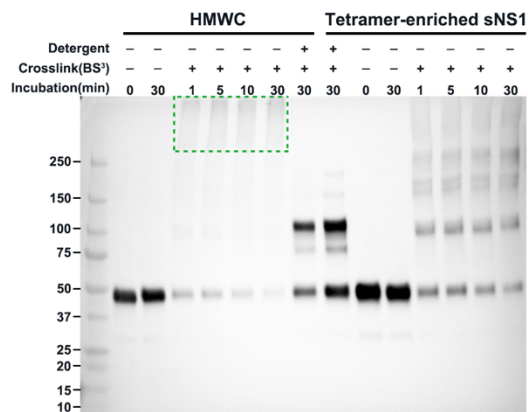
d Denaturing SDS-PAGE (Coomassie stained)



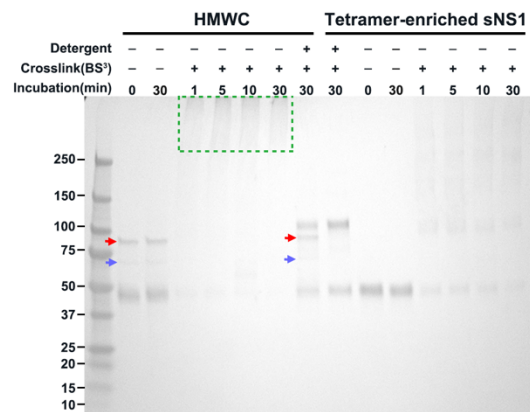
e Semi-denaturing SDS-PAGE (anti-NS1 WB)



f Anti-NS1 WB



g Coomassie Stained



Supplementary Figure 1. Purification and characterization of DENV2 sNS1 fractions.

(a) Anion exchange chromatography (Resource Q) of recombinant DENV2 sNS1 yields two distinct elution peaks, designated RQfraction 1 and RQfraction 2.

(b–c) Gel filtration profiles of RQfraction 1 and RQfraction 2 using a Superdex 200 Increase (S200i) column. **(b)** Cryo-EM analysis of RQfraction 1 reveals that this fraction is enriched for tetrameric sNS1.

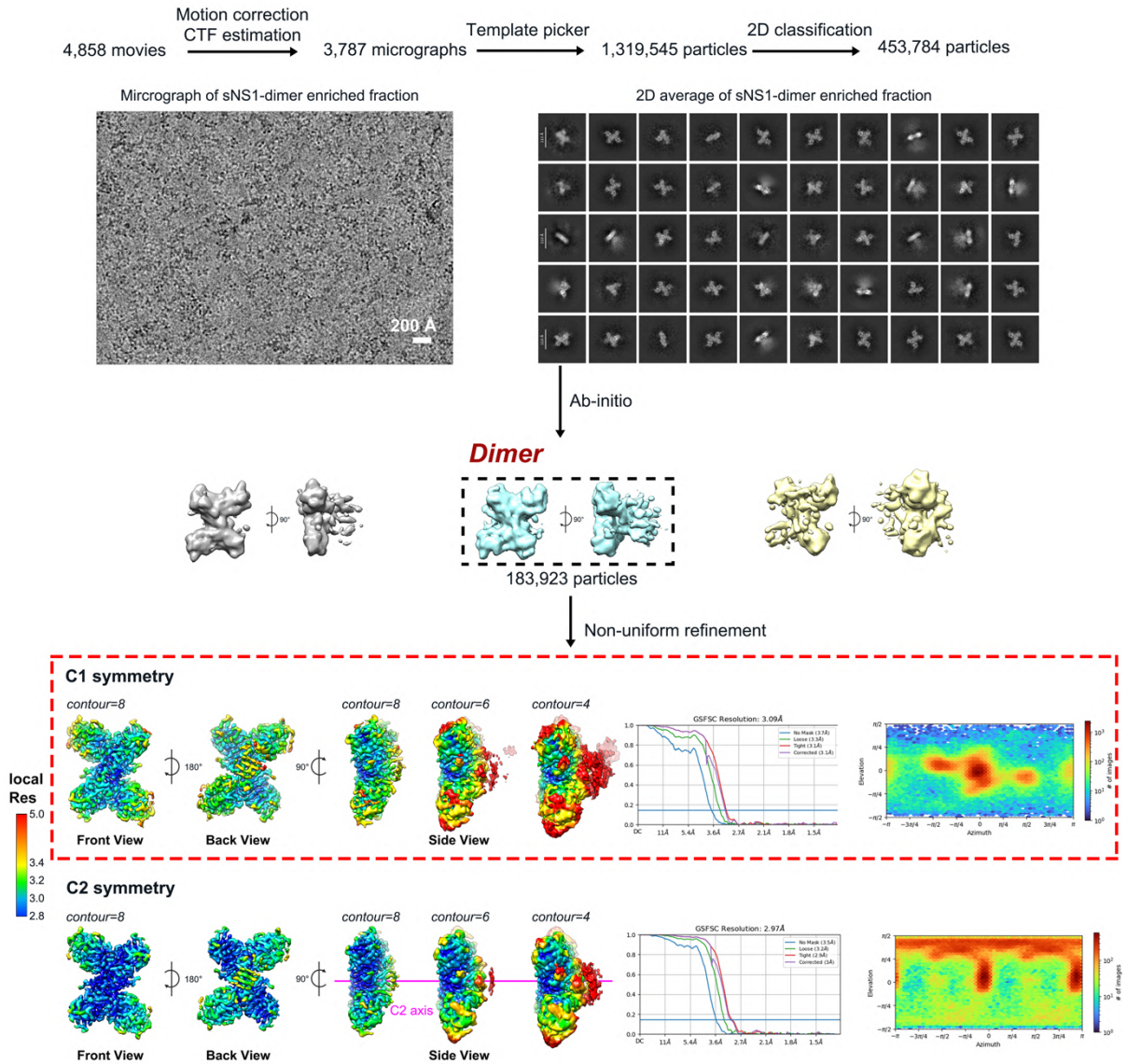
(c) RQfraction 2 eluted at a lower retention volume, indicating a higher molecular weight compared to the RQfraction 1 (tetramer-enriched fraction). The sNS1 sample eluted from RQfraction 2 is therefore referred to as the high molecular weight complex (HMWC).

(d) Denaturing SDS–PAGE analysis showed that the RQfraction 2 (HMWC) contains two additional bands at approximately 90 kDa and 70 kDa, in addition to the sNS1 band (~42 kDa), whereas the RQfraction 1 (tetramer-enriched fraction) consists predominantly of sNS1. Gel results are representative of two independent biological replicates (n=2). In-gel mass spectrometry (n=1) identified the additional bands as host chaperone proteins HSP90 (band 1) and HSP70 (band 2).

(e) Anti-NS1 Western blot analysis of the HMWC on semi-denaturing SDS–PAGE gel shows that sNS1 in the HMWC fraction forms higher-order heterogeneous assemblies (green box) compared to the tetramer-enriched fraction. Gel results are representative of two independent biological replicates (n=2).

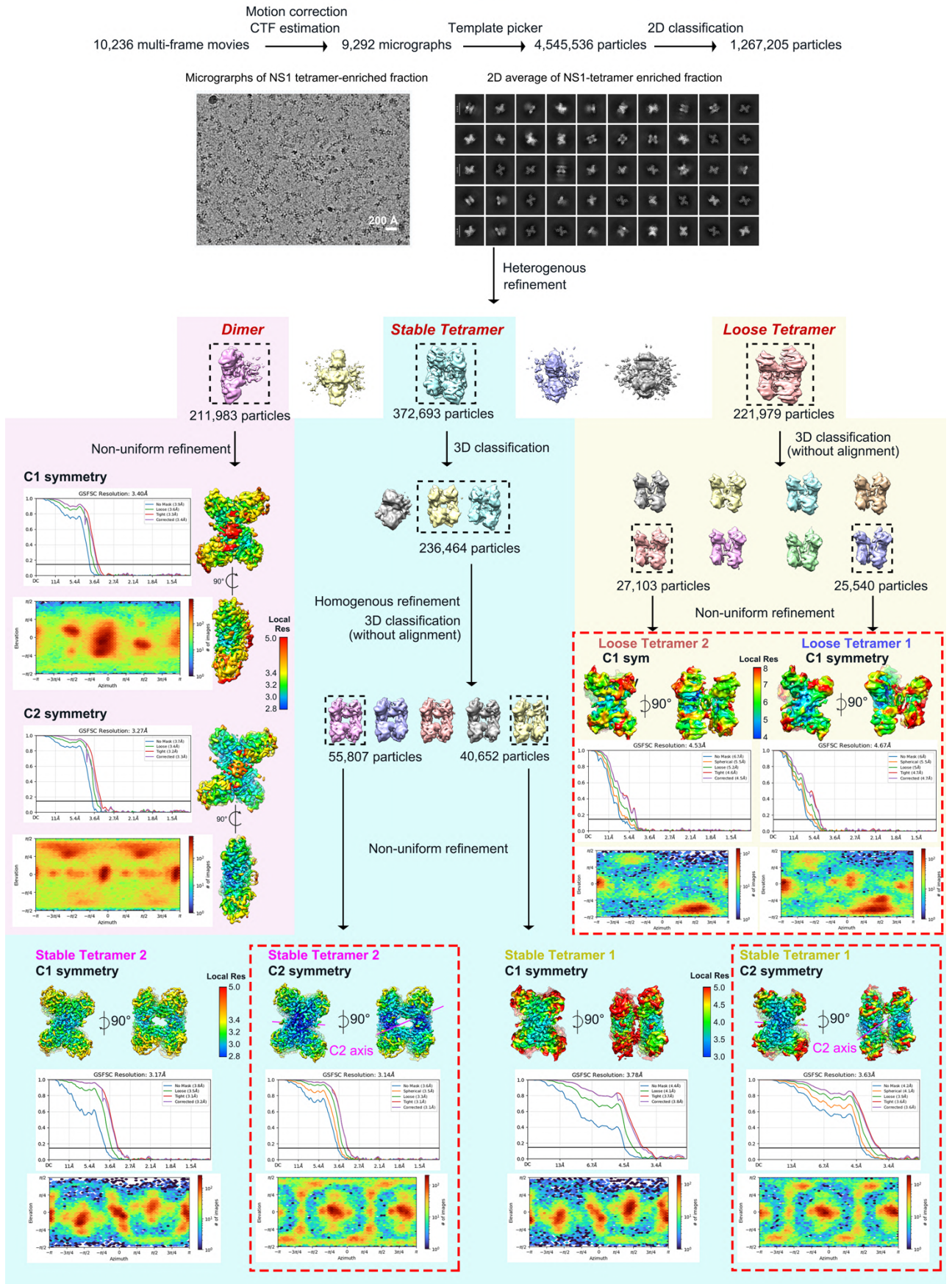
(f) Western blot analysis (anti-NS1) and **(g)** Coomassie Blue staining of the chemical crosslinking experiments performed on the HMWC and tetramer-enriched sNS1 fractions. In the absence of detergent, the HMWC fraction incubated with crosslinker shows proteins existing as large assemblies (green box). When detergent was added prior to crosslinking, the interactions between sNS1 and host chaperone proteins were disrupted (HSP90: red arrows; HSP70: blue arrows), as shown by the absence of high molecular weight complex. This suggests that the interaction between sNS1 and HSPs in the HMWC might be maintained by hydrophobic interactions. Gel results are representative of at least two independent biological replicates. Source data are provided as a Source Data file.

SPA workflow of RQfraction 2 (HMWC fraction)

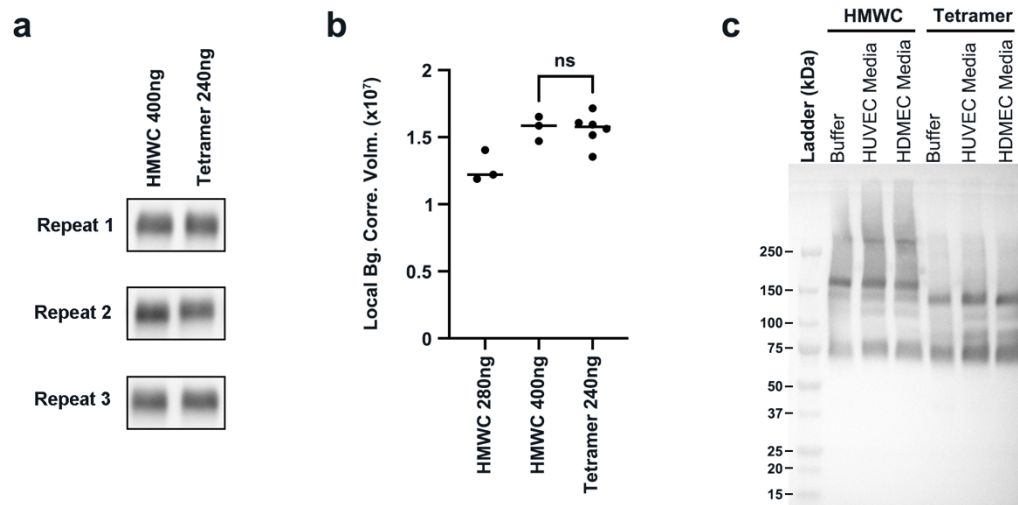


Supplementary Figure 2. Cryo-EM reconstruction workflow of DENV2 sNS1 from RQfraction 2. Cryo-EM single-particle analysis of DENV2 sNS1 (RQfraction 2) conducted using CryoSPARC. The dataset predominantly contains dimeric sNS1. Local resolution maps and global resolution estimates (based on gold-standard Fourier shell correlation, GSFSC) were calculated in CryoSPARC. The final structure deposited in the PDB/EMDB is indicated by a red dashed box.

SPA workflow of RQfraction 1 (tetramer-enriched sNS1 fraction)



Supplementary Figure 3. Cryo-EM reconstruction workflow of DENV2 sNS1 from RQfraction 1. Cryo-EM single-particle analysis of DENV2 sNS1 (RQfraction 1) performed using CryoSPARC. Local resolution maps and global resolution estimates were calculated based on gold-standard Fourier shell correlation (GSFSC). RQfraction 1 primarily contains tetrameric sNS1, with dimers accounting for approximately 26.6% of the selected particles (including dimers, stable tetramers, and loose tetramers). Final structures deposited in the PDB/EMDB are highlighted with red dashed boxes.



Supplementary Figure 4. Dilution of HMWC and tetramer-enriched fractions in buffer and different endothelial cell media does not change the sNS1 oligomerization states.

(a) Standardization of the amount of sNS1 in the HMWC fraction and tetramer-enriched fraction. A total protein input of 400 ng from the HMWC fraction produces an sNS1 band intensity comparable to 240 ng from the tetramer-enriched fraction.

(b) Quantification of band volume using local background–corrected intensity confirms that 400 ng of HMWC fraction contains an amount of sNS1 equivalent to 240 ng of tetramer-enriched fraction.

(c) Dilution of HMWC and tetramer-enriched fractions to the concentration used in the endothelial hyperpermeability assay, either in buffer or endothelial cell culture media, does not change the sNS1 oligomerization profile compared with the undiluted fractions used for cryo-EM studies (Supplementary Figure 1e), as assessed by semi-denaturing SDS-PAGE. NS1 was detected by using Western blot (anti-NS1 antibody).

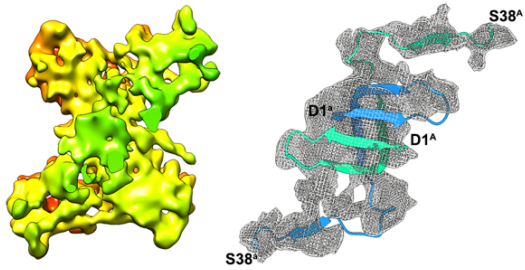
Statistics were derived from independent experiments. For panels (a–b), band intensities were quantified from three independently prepared biological samples with different dilutions resolved on the same gel (n=3). Statistical significance was assessed using two-way ANOVA with Tukey’s multiple comparisons test. Not significant (ns): p=0.9133.

For panel (c), results are representative of two independent biological replicates (n=2).

Source data are provided as a Source Data file.

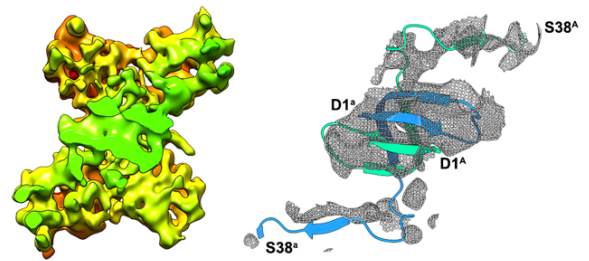
a Loose tetramer 1

(i) Map cropped through central region (ii) Fitted density



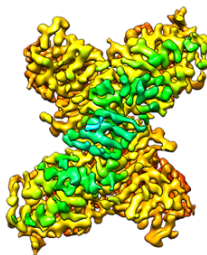
b Loose tetramer 2

(i) Map cropped through central region (ii) Fitted density

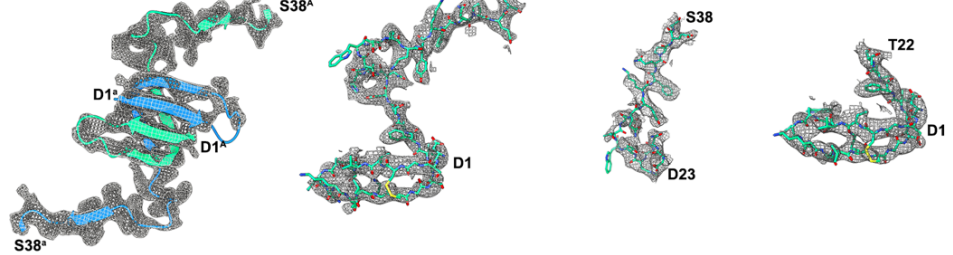


c Dimer

(i) Map



(ii) Fitted density

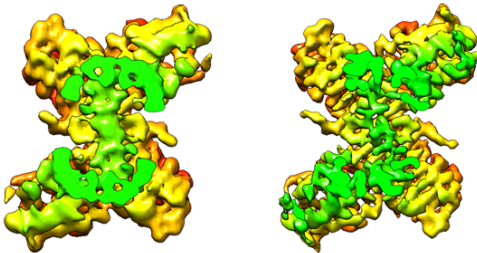


d Stable tetramer 1

(i) Maps cropped through central region

Previous published map
(Bo Shu, et. al)

New Map

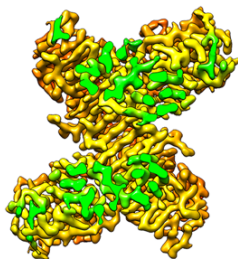


(ii) Fitted density

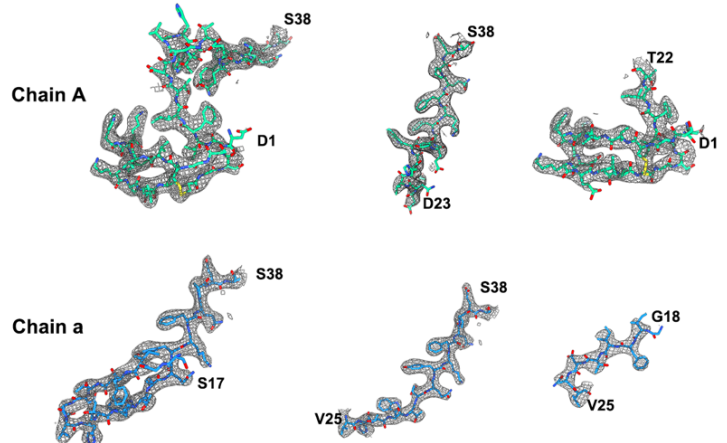


e Stable tetramer 2

(i) Map cropped through central region



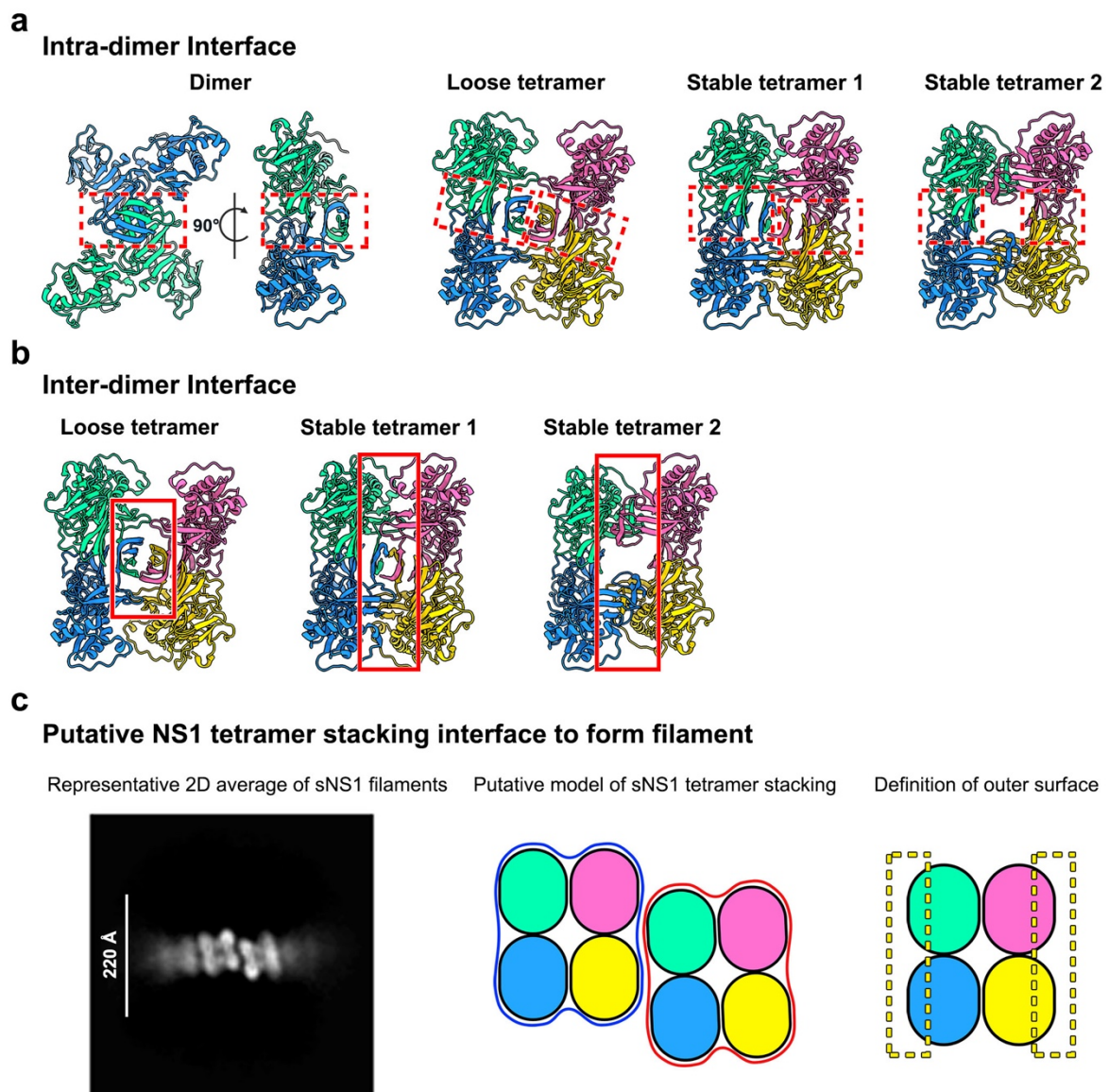
(ii) Fitted density



Supplementary Figure 5. Fitting of all oligomeric forms of sNS1 into their cryo-EM maps.

Fitting of sNS1 structures into the cryo-EM density maps of (a) Loose tetramer 1, (b) Loose tetramer 2, (c) Dimer, (d) Stable tetramer 1, and (e) Stable tetramer 2.

(i) Surface representations of all cryo-EM maps are colored by height using UCSF Chimera. In all structures, portions of the green density correspond to the $\beta 1$ – $\beta 3$ region. For Stable tetramer 1, a previously published cryo-EM map (Bo Shu et al.¹) is shown for comparison, highlighting the resolution improvement achieved in this study. (ii) Zoom-in views show the $\beta 1$ – $\beta 3$ region (residues 1–38) fitted into the cryo-EM density (shown as mesh).



Supplementary Figure 6. Definition of intra-dimer and inter-dimer $\beta 1$ – $\beta 3$ interfaces in sNS1 oligomers and the putative arrangement of tetramers in filamentous assemblies.

(a) Intra-dimer $\beta 1$ – $\beta 3$ interactions are highlighted with red dashed boxes.

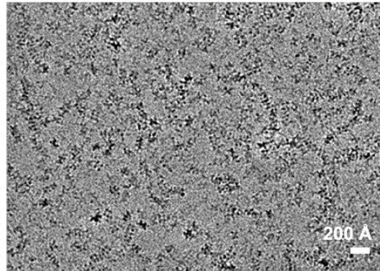
(b) Inter-dimer $\beta 1$ – $\beta 3$ interactions are marked with solid red boxes.

(c) Left: 2D class average of the filament-like assembly reveals tetramers stacking via their outer surfaces. Middle: Putative model illustrating how two tetramers interact through their outer surfaces. Right: Definition and orientation of the outer surface of a tetramer.

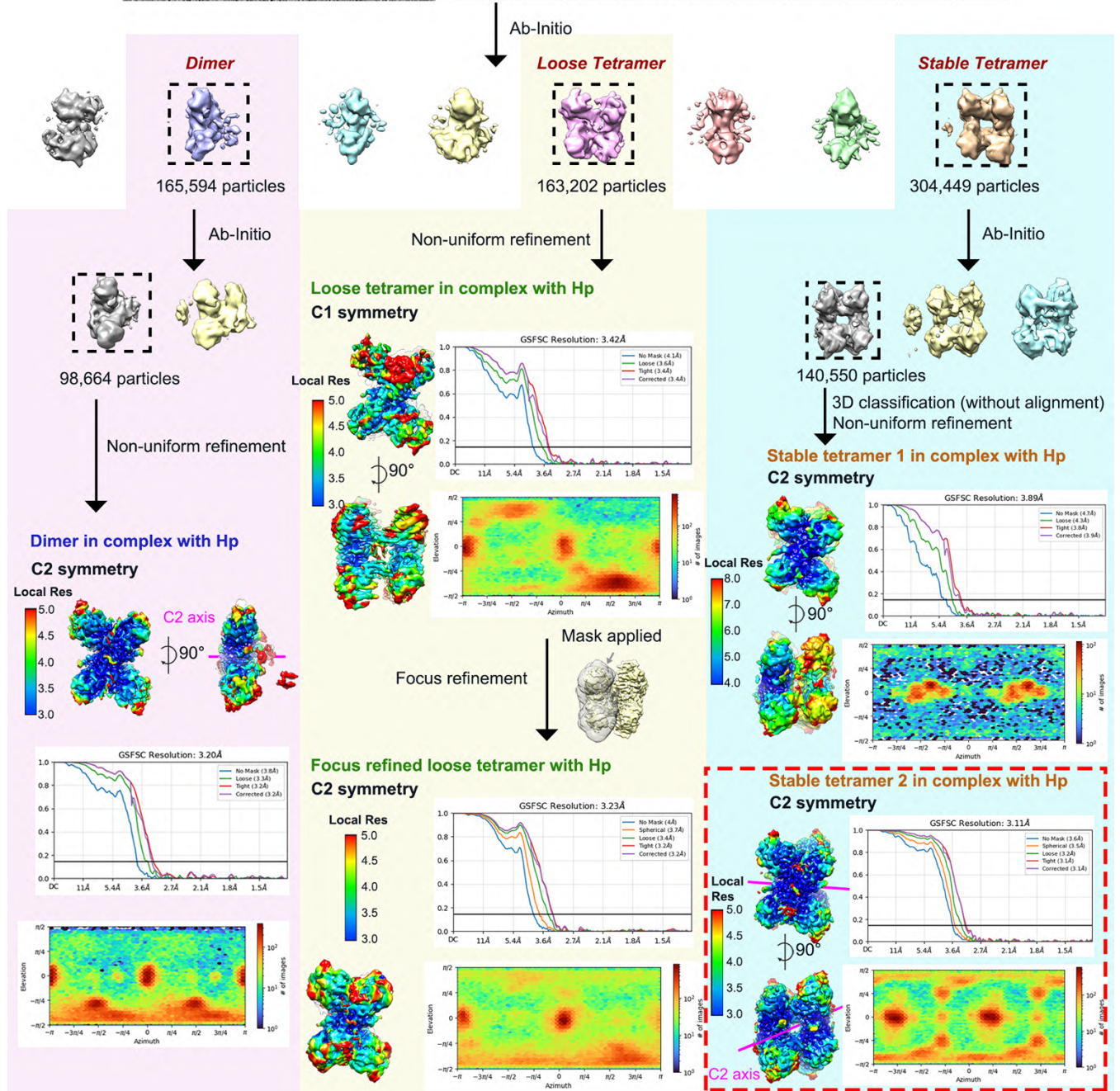
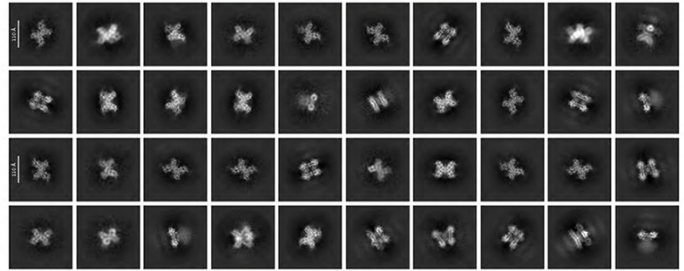
SPA workflow of NS1:Heparin complex



Micrograph of NS1-Heparin complex

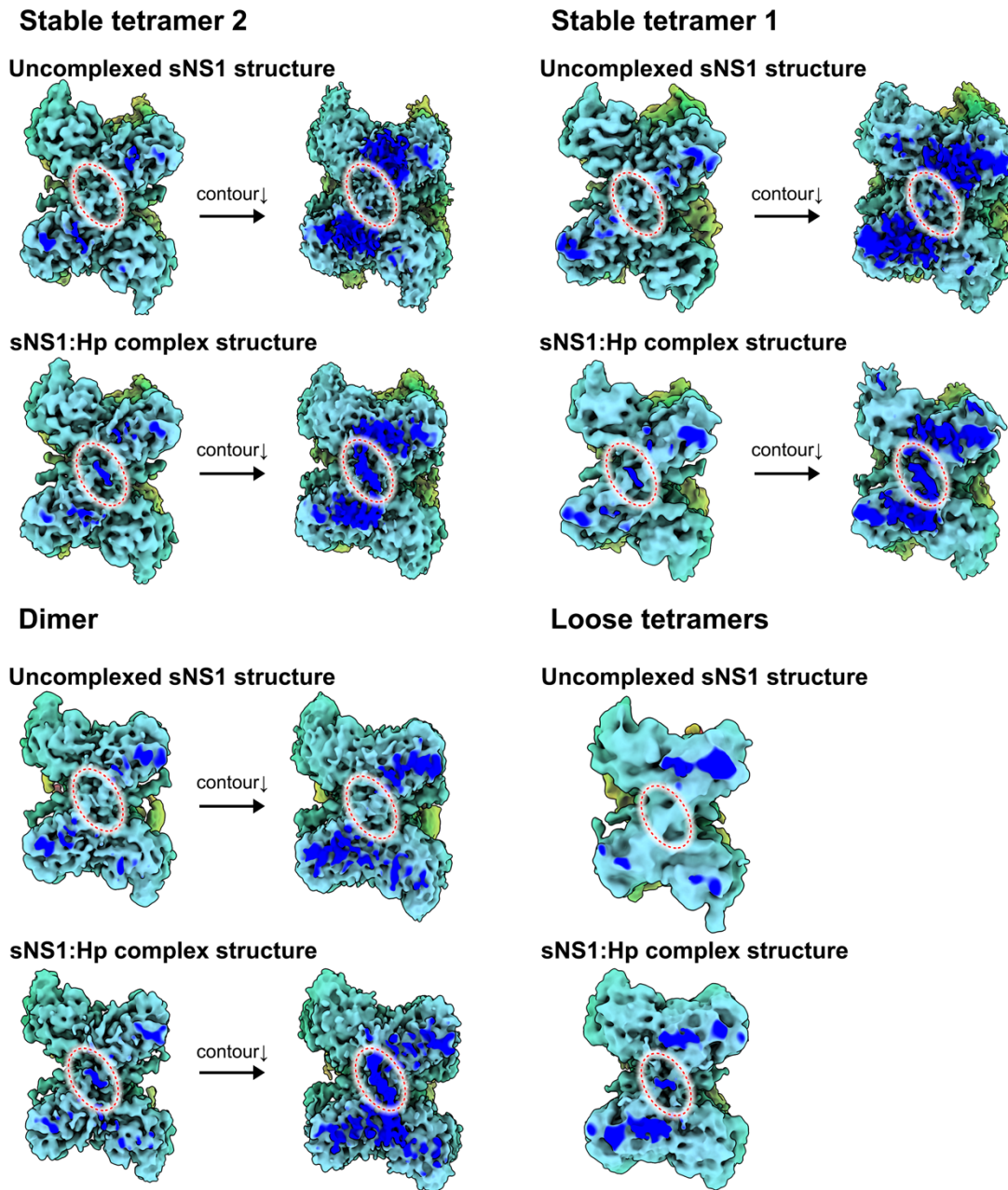


2D average of NS1-Heparin complex



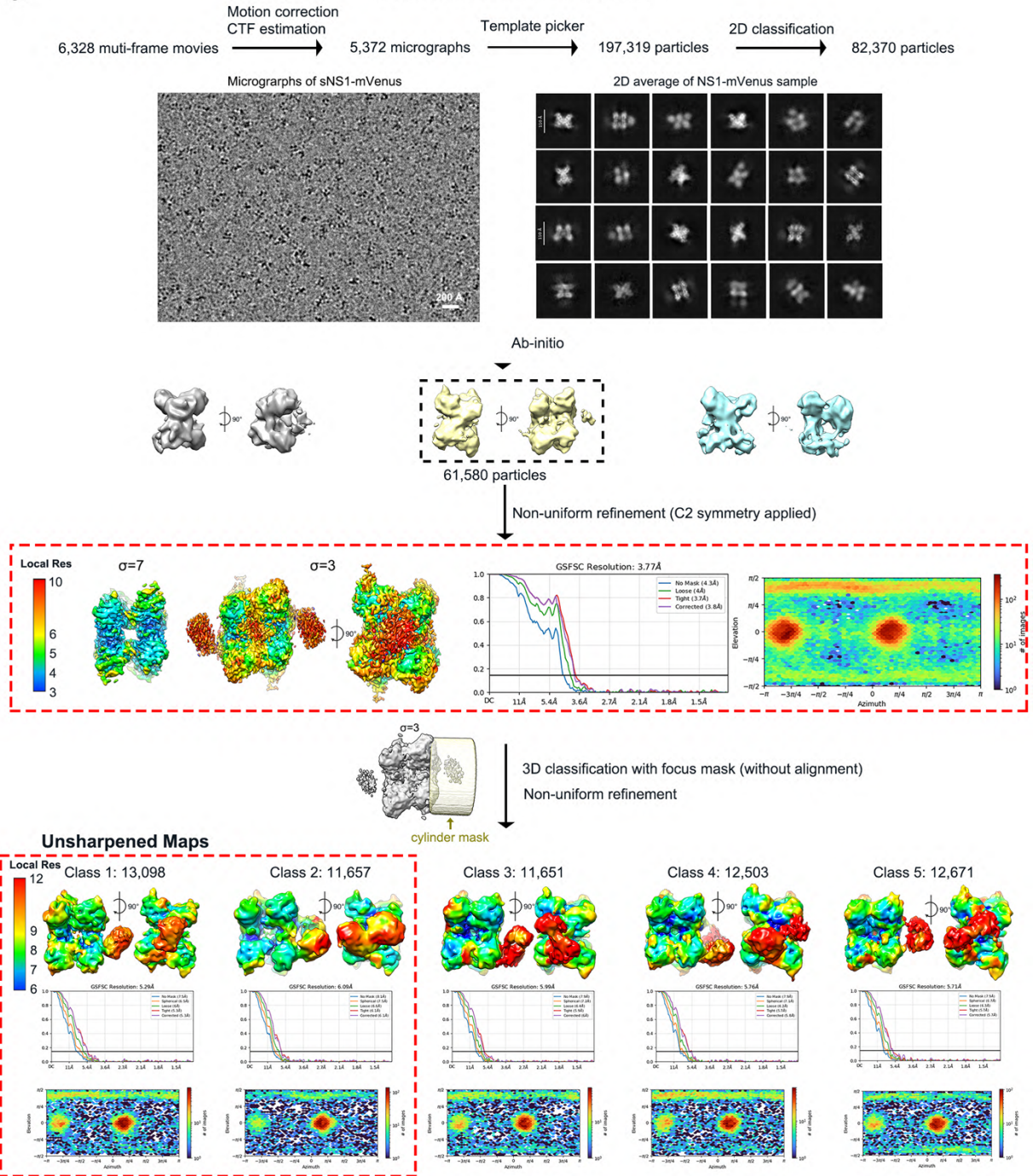
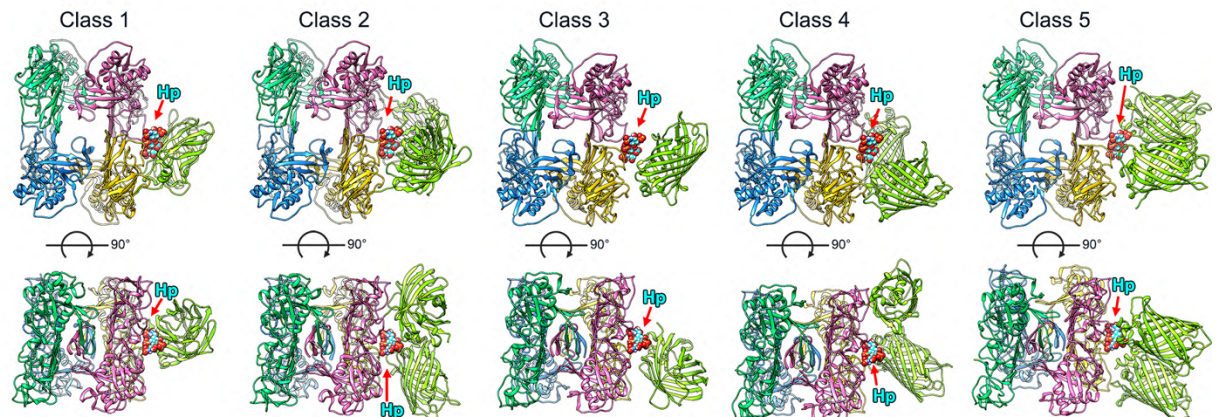
Supplementary Figure 7. Cryo-EM reconstruction workflow of DENV2 sNS1 complexed with heparin.

Cryo-EM single particle analysis workflow of the mixed oligomeric fraction of DENV2 sNS1 in complex with heparin, processed using CryoSPARC. Local resolution maps and overall resolutions were determined by gold standard Fourier shell correlation (GSFSC). Final structure deposited in the PDB/EMDB is indicated with red dashed boxes.



Supplementary Figure 8. Heparin binds to all oligomeric forms at the outer surface of the intra-dimer interface.

Cryo-EM density maps of uncomplexed sNS1 and sNS1 complexed with heparin (Hp). The red dashed line marks the outer surface of the intra-dimer interface. Maps are colored by height using Chimera² and shown at appropriate contour levels. Additional density corresponding to Hp is observed at the intra-dimer interface across all oligomeric states.

a**SPA workflow of sNS1-mVenus****b****Structural models of sNS1-mVenus classes with superimposed Hp**

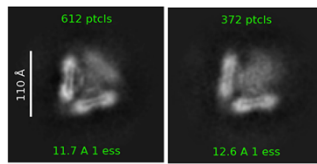
Supplementary Figure 9. Cryo-EM reconstruction workflow of DENV2 sNS1–mVenus.

(a) Cryo-EM single particle analysis workflow of sNS1–mVenus, processed using CryoSPARC. Final structures deposited in the PDB/EMDB are indicated with red dashed boxes.

(b) Five structural classes were resolved, showing varying positions of the mVenus tag relative to the sNS1 tetramer. Superimposition of the sNS1 tetramer:Hp complex suggests that the presence of mVenus may introduce steric hindrance, potentially blocking Hp (represented as a sphere) from binding.

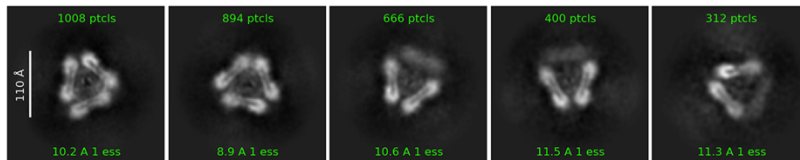
a HMWC Fraction

Putative hexamer-like sNS1 2D class averages



b Tetramer-enriched sNS1 Fraction

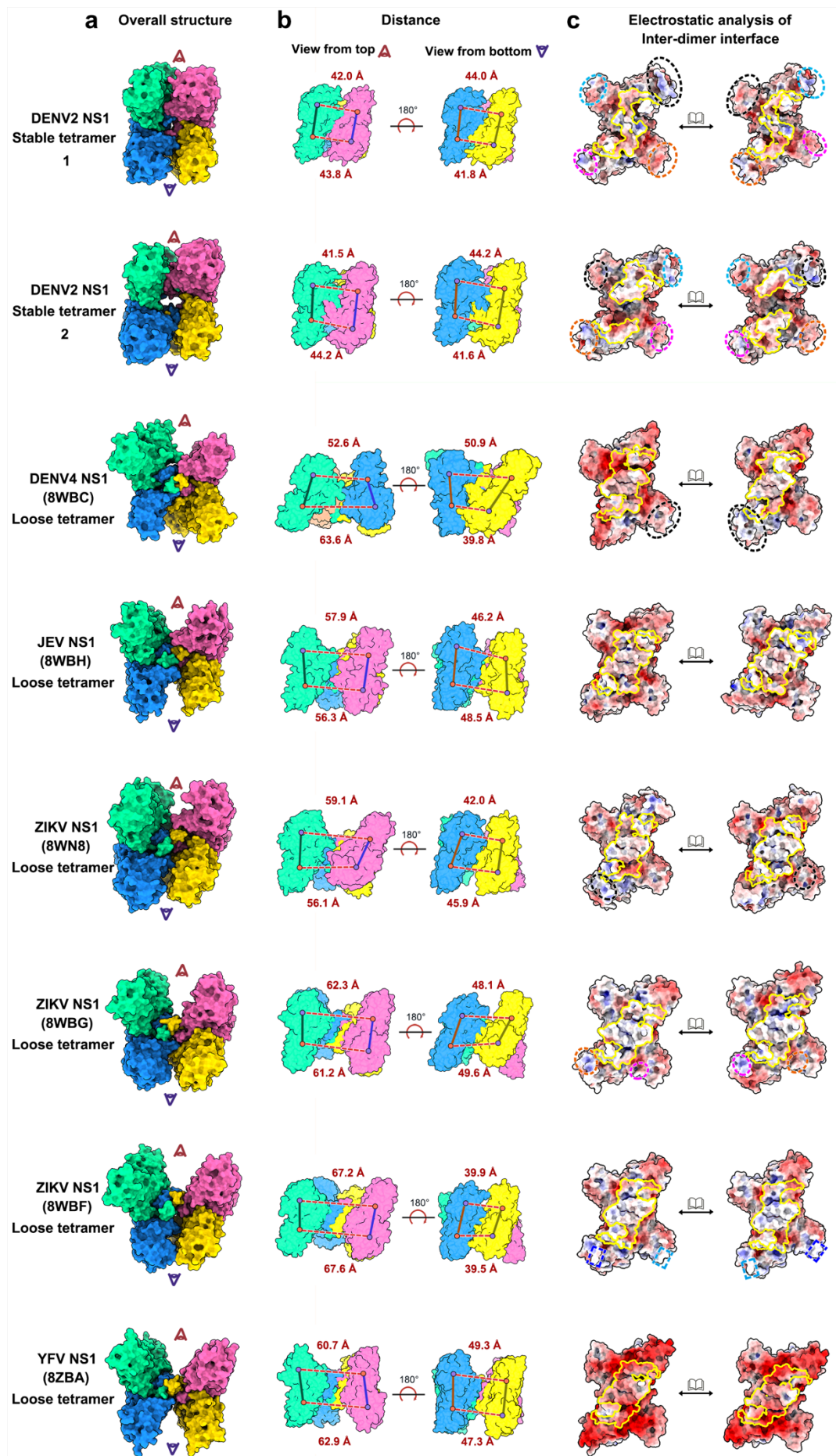
Putative hexamer-like sNS1 2D class averages



Supplementary Figure 10. Observation of hexamer-like particles in DENV2 sNS1 preparations.

(a) In the HMWC fraction, extensive 2D classification identified fewer than 1,000 particles that may correspond to hexamer-like assemblies (left).

(b) In the tetramer-enriched sNS1 fraction, fewer than 1% of particles were classified as hexamer-like. The dimer units within the hexamer-like particles show flexible and heterogeneous arrangements.

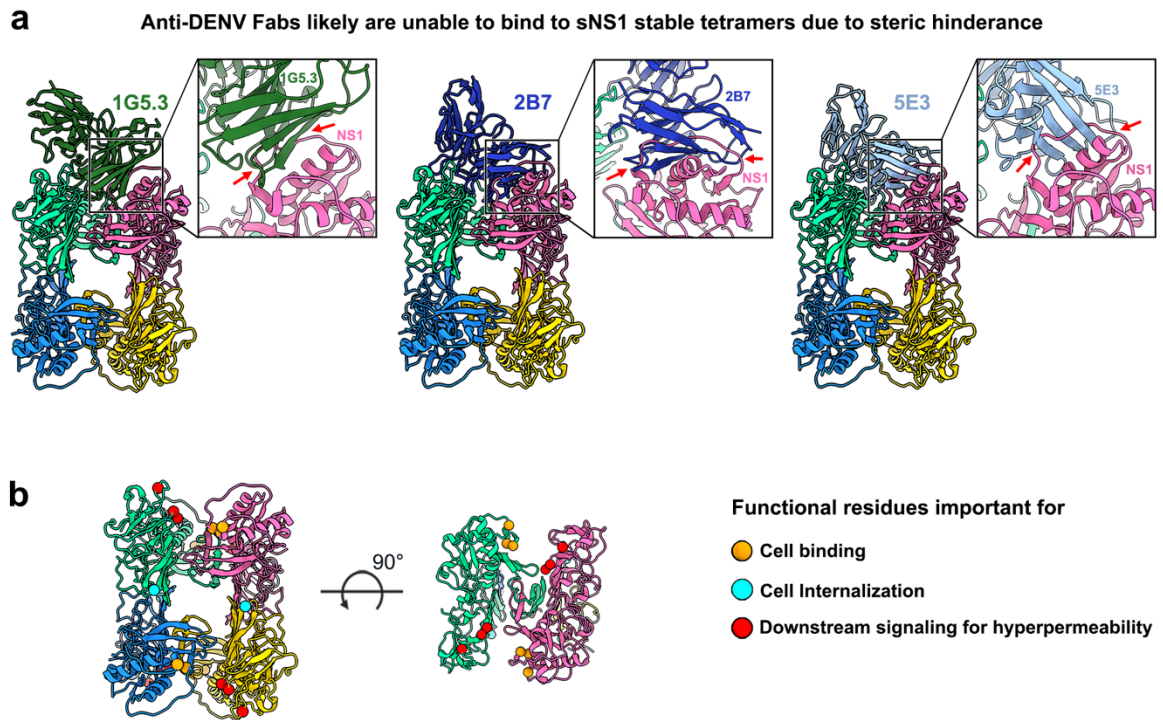


Supplementary Figure 11. Electrostatic surface charges of the inter-dimer interface within sNS1 tetramers across published flaviviruses³⁻⁵.

(a) Side view of sNS1 tetramers from various flaviviruses; only DENV2 PVP94/07 forms a stable tetramer.

(b) Top and bottom views of the tetramers showing distances between protomers at the inter-dimer interface. Centroids of the wing domain (purple circles) and β -ladder domain (orange circles) are indicated for each NS1 protomers. Solid lines connect the wing and β -ladder centroids within each protomer, while red dashed lines represent inter-dimer centroid distances, reflecting inter-protomer interactions within the tetramer.

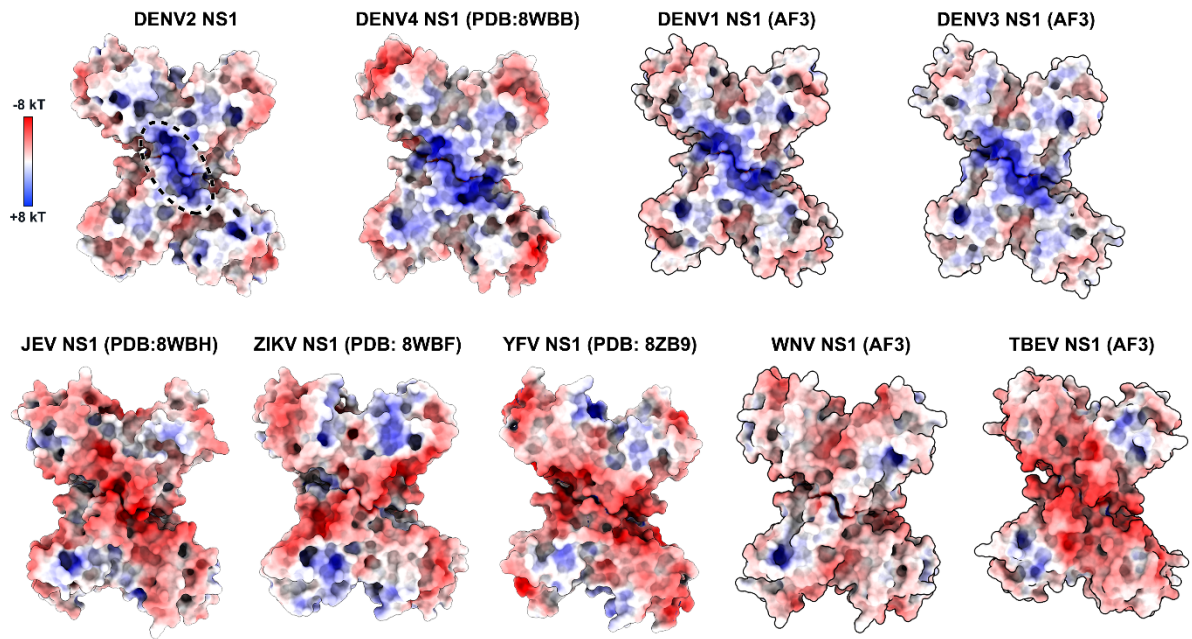
(c) Open-book representation of electrostatic surface charges on the inter-dimer interface within the tetramers shown in (a). DENV2 PVP94/07 displays the most complementary charge distribution. The boundary of the N-terminal β 1– β 3 region (residues 1–37) is outlined in yellow. Complementary charged regions are circled with dotted lines matching their colors. Surface potentials range from electropositive (blue, +8 kT) to electronegative (red, –8 kT).



Supplementary Figure 12. Antibody binding sites and functional residues mapped onto the stable tetramer 2 structure of NS1.

(a) Superimposition of DENV anti-NS1 Fabs (1G5.3⁶, 2B7⁷ and 5E3¹) complexed with a dimer onto one protomer within the stable tetramer 2 structure. The model suggests that these Fabs may be sterically hindered from binding (indicated by red arrow).

(b) Mapping of previously identified residues critical for NS1 binding⁷, cellular internalization⁸, and downstream signaling⁷ that leads to endothelial hyperpermeability, shown as spheres on the stable tetramer 2 structure.



Supplementary Figure 13. Electrostatic analysis of the outer surface of NS1 dimers from various flaviviruses indicates that only DENV NS1 binds heparin (Hp) at the basic groove in the intra-dimer interface.

Hp binding occurs at the basic groove (highlighted by a dotted black circle) in our sNS1 structures. This groove is observed exclusively in DENV1–4 NS1. For DENV2, DENV4, JEV, ZIKV, and YFV^{3,4}, electrostatic surface maps were calculated from experimentally determined structures (PDB IDs are indicated). For DENV1, DENV3, WNV, and TBEV, NS1 dimer models were generated using AlphaFold 3 (AF3)⁹. Structures modeled by AF3 are labeled with “(AF3)” in the figure.

Supplementary Table 1. Top reads of LC–MS/MS analysis of proteins co-purified with NS1 fractions

Protein	Uniprot Accession	Gene	#Peptides	Area HMWC Fraction	Area Tetramer- enriched Fraction	#Spec HMWC Fraction	#Spec Tetramer- enriched Fraction	MW (kDa)
NS1 (DENV2)	H9M645	POLY	56	4.70E+10	7.06E+10	1051	1444	40.8
HSP90 α	P07900	HSP90AA1	80	7.12E+09	9.55E+06	891	46	85
HSP90 β	P08238	HSP90AB1	76	2.85E+09	4.39E+06	601	34	83
HSP70-1A	P0DMV9	HSPA1B	59	2.54E+09	1.51E+07	394	47	70
HSP70-1B	P0DMV8	HSPA1A	59	2.54E+09	1.51E+07	394	47	70

Supplementary Table 2. Statistics of data collection, data process, model refinement, and validation

Sample	sNS1 Dimer	sNS1 Loose Tetramer 1	sNS1 Loose Tetramer 2	sNS1 Stable Tetramer 1	sNS1 Stable Tetramer 2	sNS1+Heparin Stable Tetramer 2	sNS1- mVenus	sNS1- mVenus Class1	sNS1- mVenus Class2
EMDB	65563	65562	65558	65561	65560	65564	65565	65566	65559
PDB code	9W22	9W21	9W1X	9W20	9W1Z	9W23	9W24	9W25	9W1Y
Data collection									
Magnification	130,000	130,000	130,000	130,000	130,000	130,000	130,000	130,000	130,000
Voltage (kV)	300	300	300	300	300	300	300	300	300
Electron exposure (e ⁻ / (Å ²))	78.5	80.3	80.3	80.3	80.3	80.2	79.8	79.8	79.8
Defocus range (μm)	-1.0 to - 2.2	-1.0 to -2.2	-1.0 to -2.2	-1.0 to -2.2	-1.0 to -2.2	-1.0 to -2.2	-1.0 to -2.2	-1.0 to -2.2	-1.0 to -2.2
Pixel size (Å)	0.671	0.671	0.671	0.671	0.671	0.671	0.671	0.671	0.671
3D Reconstruction									
# of Micrographs	4,858	10,236	10,236	10,236	10,236	9,464	6,328	6,328	6,328
# of particles picked	1,319,5 45	4,545,536	4,545,536	4,545,536	4,545,536	3,697,637	197,319	197,319	197,319
# of particles for final refinement	183,923	25,540	27,103	40,652	55,807	304,449	61,580	13,098	11,657
Symmetry imposed	C1	C1	C1	C2	C2	C2	C2	C1	C1
Map sharpening B-factor (Å ²)	-90	-120	-100	-80	-80	-20	-140	-	-
Resolution FSC=0.143 (Å)	3.1	4.7	4.5	3.6	3.1	3.1	3.8	5.3	6.1
Model Refinement									
Model composition									
Refinement scope	Full- atom	Full-atom	Full-atom	Full-atom	Full-atom	Full-atom	Full-atom	Ca backbone	Ca backbone
# of chains	2	4	4	4	4	6	4	4	4
# of residues	692	1384	1384	1353	1376	1376	1376	1612	1848
# of atoms	5532	11064	11064	10799	10988	11150	10988	1612	1848
# of ligands	-	-	-	-	-	2	-	-	-
Validation									
MolProbity score	1.95	1.82	1.80	2.49	2.10	2.48	2.24	1.99	2.06
All-atom clash score	6.60	13.01	14.80	10.24	5.17	20.42	7.20	1.24	1.62

Rotamer outliers (%)	2.96	0.00	0.00	4.22	3.56	2.15	3.73	NA	NA
Ramachandran Plot values									
Favoured (%)	96.51	96.80	97.38	91.97	93.93	93.49	93.79	NA	NA
Allowed (%)	3.34	3.20	2.62	7.96	6.07	6.51	6.21	NA	NA
Outliers (%)	0.15	0.00	0.00	0.07	0.00	0.00	0.00	NA	NA
R.M.S Deviations									
Bond lengths (Å)	0.004	0.002	0.002	0.003	0.005	0.004	0.002	NA	NA
Bond angles	0.570	0.621	0.594	0.549	0.646	0.454	0.479	NA	NA
Correlation Coefficient (mean± std)									
Protein	0.78±0.01	0.59 ± 0.11	0.63±0.04	0.79±0.01	0.764 ± 0.002	0.889 ± 0.002	0.61±0.013	0.61±0.09	0.60±0.06
Ligand	-	-	-	-	-	0.45 ± 0.0004	-	-	-

Supplementary Methods

Materials

For Western blot detection of NS1, a human monoclonal antibody 5E3 (anti-DENV2 NS1) was used as the primary antibody. The antibody was isolated and purified in the laboratory of Gavin Screaton. Goat anti-Human IgG (H+L) Secondary Antibody, HRP (Life Technologies, catalog #H10307) was used as the secondary antibody. Antibodies were used at the following dilutions: primary antibody 1 µg/mL, secondary antibody 1:2000.

Crosslinking assay (Supplementary Figure 1f-g)

sNS1 samples from HMWC fraction and tetramer-enriched fraction were buffer-exchanged into conjugation buffer (PBS) using PD-10 desalting columns and adjusted to a final concentration of 0.03 mg/mL. The amine-reactive crosslinker bis(sulfosuccinimidyl) suberate (BS³) was prepared at 100 mM in water according to the manufacturer's instructions and subsequently diluted to 20 mM in PBS immediately before use.

For each reaction, 20 µL of sNS1 sample was mixed with 1 µL of BS³ to achieve a final BS³ concentration of approximately 1 mM. Control reactions received 1 µL of PBS instead of BS³. Crosslinking reactions were incubated at room temperature for 1, 5, 10, or 30 min and then quenched by addition of 1 µL of 1 M Tris-HCl (pH 7.4), yielding a final Tris concentration of approximately 50 mM. Quenching was allowed to proceed for 15 min at room temperature.

To disrupt protein-protein interactions prior to crosslinking, 1 µL of 2% SDS was added to sNS1 samples and incubated for 10 min at room temperature before addition of BS³.

Reaction products were analyzed by SDS-PAGE followed by Western blotting using an anti-NS1 monoclonal antibody (5E3) to detect crosslinked products.

Calibration of sNS1 content in HMWC fraction and tetramer-enriched fraction (Supplementary Figure 4a-b)

To calibrate the relative amount of sNS1 in the HMWC fraction and the tetramer-enriched fraction, 3.5 µg or 5 µg of total protein from the HMWC fraction, or 3 µg of total protein from the tetramer-enriched fraction, were diluted to a final volume of 70 µL in buffer. Reducing SDS loading dye (30 µL) was added to each sample, followed by boiling at 95 °C for 10 min. From each mixture, 8 µL was loaded onto an SDS-PAGE gel, corresponding to final loaded amounts of 280 ng HMWC fraction, 400 ng HMWC fraction, and 240 ng tetramer-enriched fraction, respectively. Proteins were resolved by SDS-PAGE and transferred for Western blot analysis using an anti-NS1 monoclonal antibody (5E3) to detect sNS1. Band intensities corresponding to sNS1 were quantified using an iBright 1500 imaging system (Invitrogen) and are reported as background-subtracted integrated band volumes (local background corrected volumes).

Analysis of sNS1 oligomerization status in endothelial cell media by non-reducing SDS-PAGE (Supplementary Figure 4c)

To assess the oligomerization status of sNS1 in endothelial cell culture media, 5 µg of total protein from the HMWC sNS1 assemblies fraction or 3 µg of total protein from the tetramer-enriched sNS1 fraction were diluted into a final volume of 300 µL of either NS1 buffer (150 mM NaCl, 20 mM Tris-HCl, pH 8.0), HUVEC culture medium (EGM-2, Lonza), or HDMEC culture medium (EGM-2 MV, Lonza).

Samples diluted in endothelial cell media were incubated at 37 °C for 30 min, whereas samples diluted in NS1 buffer were kept on ice for 30 min. Following incubation, 20 µL of each sample was mixed with 8 µL of non-reducing SDS loading dye. A total of 15 µL of each prepared sample was then resolved by SDS–PAGE, followed by Western blot analysis using an anti-NS1 monoclonal antibody (5E3) to evaluate the oligomeric status of sNS1.

Proteomics Liquid chromatography–tandem mass spectrometry (LC–MS/MS) (Supplementary Table 1)

Protein samples from HMWC fraction (0.3mg/ml, n=1 biological replicate) and tetramer-enriched fraction (0.8mg/ml, n=1 biological replicate) were lysed in S-TRAP lysis buffer (50 mM triethylammonium bicarbonate, 5% SDS) and processed using S-TRAP Micro columns (Protifi) according to the manufacturer's instructions. Proteins were digested on-column, and resulting peptides were recovered for LC–MS/MS analysis. NS1 buffer was used as the control.

Peptide separation was performed using an EvoSep One system (EvoSep) equipped with an Ionopticks Aurora Elite XT C18 analytical column (1.7 µm particle size, 75 µm × 150 mm). Peptides were eluted using a binary solvent system consisting of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile), operated using the Whisper Zoom 40 samples-per-day method.

Mass spectrometric data were acquired on a Thermo Fisher Scientific Orbitrap Exploris 480 mass spectrometer operated in positive ion mode with a spray voltage of 1.5 kV and an ion transfer tube temperature of 305 °C. Full MS scans were acquired in the Orbitrap over a mass range of m/z 400–1600 at a resolution of 60,000, with an automatic gain control (AGC) target of 3×10^6 and a maximum injection time of 25 ms. The RF lens was set to 50%. MS/MS spectra were acquired using data-dependent acquisition with a cycle time of 2 s and dynamic exclusion enabled. Precursor ions with charge states of 2–6 and intensities above 1×10^5 were selected for fragmentation using higher-energy collisional dissociation (HCD) with a normalized collision energy of 30%. Fragment ions were analyzed in the Orbitrap at a resolution of 15,000, using an isolation window of 1.6 m/z, an AGC target of 5×10^5 , and a first mass of m/z 100.

Raw data were processed using PEAKS Studio (version 13.0; Bioinformatics Solutions Inc.). Spectra were searched against the Swiss-Prot Homo sapiens database (release 2025_03), a universal contaminants database, and the Flavivirus (9FLAV) genome polyprotein sequence database. Search parameters included a precursor mass tolerance of 10 ppm and a fragment mass tolerance of 0.02 Da, allowing up to two missed cleavages. Peptides of 6–45 amino acids were considered. Oxidation of methionine (+15.99 Da) was included as a fixed modification, while N-terminal acetylation (+42.01 Da), phosphorylation of serine, threonine, and tyrosine (+79.97 Da), and carbamidomethylation (+57.02 Da) were set as variable modifications, with a maximum of two variable modifications per peptide. Protein identifications were filtered to a false discovery rate (FDR) of 1.0% at both the peptide-spectrum match and protein group levels, requiring at least one unique peptide per protein.

In-gel protein identification and LC–MS/MS analysis (Supplementary Figure 1d)

SDS–PAGE was performed to separate protein components in the HMWC fraction (n=1 biological replicate). Protein bands at approximately ~70 kDa and ~90 kDa were excised from the gel and cut into ~1 mm³ pieces (Supplementary Figure 1d). NS1 buffer was used as the control. Gel pieces were transferred to a Sep-Pak tC18 96-well µElution Plate (Waters, Milford, MA, USA), washed with

50 mM ammonium bicarbonate in 50% ethanol (v/v), and dehydrated with 100% ethanol. This wash–dehydration step was repeated once. Proteins were reduced with 10 mM dithiothreitol at 56 °C for 60 min and subsequently alkylated with 55 mM iodoacetamide for 60 min at room temperature in the dark. Gel pieces were then sequentially washed with 200 µL of 50 mM ammonium bicarbonate (30 min), 200 µL of 25 mM ammonium bicarbonate containing 5% acetonitrile (30 min), and 200 µL of 25 mM ammonium bicarbonate containing 50% acetonitrile (30 min), followed by dehydration with 200 µL acetonitrile for 10 min. Trypsin digestion was performed by adding 1 µg of trypsin per 20 µg of protein and incubating at 37 °C for 18 h. Following digestion, gel pieces were washed with 0.2% trifluoroacetic acid (TFA), and peptides were extracted using 70% acetonitrile containing 0.1% TFA.

Peptide separation was carried out using an Eksigent nanoLC Ultra system coupled with a ChiPLC-nanoflex platform (Eksigent) operated in trap–elute configuration. Peptides were desalted using a Sep-Pak tC18 µElution Plate (Waters) and reconstituted in 20 µL of diluent (98% water, 2% acetonitrile, 0.05% formic acid). A 5 µL aliquot of each sample was loaded onto a 200 µm × 0.5 mm trap column and separated on an analytical column (75 µm × 150 mm). Both columns were packed with ChromXP C18-CL resin (3 µm; Eksigent).

Peptides were eluted at a flow rate of 300 nL/min using a gradient of mobile phase A (2% acetonitrile, 0.1% formic acid) and mobile phase B (98% acetonitrile, 0.1% formic acid) as follows: 5–30% B over 10 min, 30–60% B over 4 min, 60–90% B over 1 min, 90% B for 5 min, 90–5% B over 1 min, and 5% B for 9 min.

Peptide identification and quantification were performed using ProteinPilot software (version 5.0.3.0, revision 5313; SCIEX) with the Paragon database search algorithm (version 5.0.3.0.5312) and integrated false discovery rate (FDR) analysis. Spectra were searched against the Swiss-Prot human database (20,456 entries). Search parameters included: sample type, identification; cysteine alkylation, iodoacetamide; digestion, trypsin; special factors, gel-based ID; species, none. Data processing was performed with ID focus set to biological modifications, search effort set to thorough, and detected protein threshold set to 0.05 (10%).

FDR estimation was conducted using a concatenated target–decoy database with in silico sequence reversal generated automatically by the software. Only proteins identified with ≤1% global FDR and peptides with ≤5% local FDR were considered for further analysis.

Sample preparation for lipidomic analysis and lipidomic analysis

For lipid extraction, 10 µL of the HMWC fraction (0.3 mg/mL) or 10 µL of the tetramer-enriched sNS1 fraction were each mixed with 200 µL of butanol:methanol (1:1, v/v) containing internal standards (ISTDs). NS1 buffer was used as the control. Samples were vortexed for 5 min and then centrifuged at 14,000 × g for 10 min at 4 °C. The resulting supernatants were collected and subjected to lipidomic analysis.

Untargeted lipidomic analysis was performed using supercritical fluid chromatography coupled to quadrupole time-of-flight mass spectrometry (SFC/MS-QTOF) on an Agilent 6546 LC/Q-TOF system equipped with an Agilent 1260 Infinity II SFC system. Instrumental parameters and analytical conditions were identical to those previously described¹⁰.

Supplementary References

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