

# Supporting Information

**A versatile reversible crosslinking strategy to stabilize CCMV virus like particles for efficient *siRNA* delivery**

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## 1 Materials and methods

### 1.1 Materials

Hot start II HF DNA polymerase, restriction enzymes, and T4 DNA ligases were obtained from New England Biolabs. DNA oligos were synthesized by Integrated DNA Technologies (IDT). Ampicillin and chloramphenicol, heparin sodium salt from porcine intestinal mucosa, nickel (II) chloride hexahydrate and Benzonase were obtained from Sigma-Aldrich. Isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) was purchased from UBPBio. Ni-NTA agarose beads were obtained from Qiagen. DTSSP (3,3'-dithiobis[sulfosuccinimidylpropionate]), yeast tRNA, RNase A were purchased from ThermoFisher Scientific. Luciferase GL3 Duplex was purchased from Dharmacon. Quant-iT™ RiboGreen™ RNA Reagent was obtained from Invitrogen. The Luciferase assay system was obtained from Promega.

### 1.2 Buffers

Name	Composition
Lysis buffer	50 mM NaH <sub>2</sub> PO <sub>4</sub> , 300 mM NaCl, 10 mM Imidazole, pH 8
Washing buffer	50 mM NaH <sub>2</sub> PO <sub>4</sub> , 300 mM NaCl, 20 mM Imidazole, pH 8
Elution buffer	50 mM NaH <sub>2</sub> PO <sub>4</sub> , 300 mM NaCl, 250 mM Imidazole, pH 8
Dimer buffer	50 mM Tris·HCl, 500 mM NaCl, 10 mM MgCl <sub>2</sub> , 1 mM EDTA, pH 7.5
Capsid buffer	50 mM NaOAc, 500 mM NaCl, 10 mM MgCl <sub>2</sub> , pH 5.0
PBS	137 mM NaCl, 2.7 mM KCl, 10 mM Na <sub>2</sub> HPO <sub>4</sub> , 1.8 mM KH <sub>2</sub> PO <sub>4</sub> , pH 7.2
Assembly buffer	50 mM Tris, 50 mM NaCl, 5 mM MgCl <sub>2</sub> , 10 mM KCl pH 7.2

**Table S1.** Buffer compositions, all buffers were filtered over a 0.2 micron filter and autoclaved prior to use.

### 1.3 UV-vis absorbance measurements

Protein concentration as well as the evaluation of tRNA (25000 g mol<sup>-1</sup>) and siRNA (13300 g mol<sup>-1</sup>) encapsulation were calculated from the absorbance at 280 and 260 nm, together with the theoretical extinction coefficients (625000 and 367391 (L mol<sup>-1</sup> cm<sup>-1</sup>) respectively) using a spectrophotometer ND-1000 (Peqlab Biotechnologie GmbH).

#### **1.4 Mass spectrometry**

Protein mass characterization was performed using a high resolution LC-MS system (Waters ACQUITY UPLC I-Class system coupled to Xevo G2 Quadrupole Time of Flight). Proteins were separated using a Polaris C18A reverse phase column, 2.0x100nm, Agilent. Deconvoluted mass spectra were obtained using the MaxENT1 algorithm and the Masslynx v4.1 (SCN862) software. Protein samples were desalted by spin filtration in Milli-Q water (final concentrations 0.5 g L<sup>-1</sup>) prior to measurements.

#### **1.5 Size exclusion chromatography (SEC)**

SEC measurements were performed on a Superose 6 increase 10/300 column (GE Healthcare). Analytical SEC measurements were executed on an Agilent 1260 bio-inert HPLC. Samples (final CP concentration of 1 g L<sup>-1</sup>) were separated with a flow rate of 0.5 mL/min in PBS<sub>1</sub>.

#### **1.6 Transmission electron microscopy (TEM)**

Protein samples were prepared according to a final CP concentration of 0.5 g L<sup>-1</sup>. TEM grids (FCF-200-Cu, EMS) were glow-discharged using a Cressington carbon coater and power unit. Protein samples (5 µL) were applied on the glow-discharged grids and incubated for 1 min. The samples were carefully removed using a filter paper. Finally, grids were negatively stained by incubation with 2% uranyl acetate solution (5 µL) in water for 15 seconds. After removal of the staining solutions, grids were allowed to dry for at least 15 minutes. The samples were analyzed on a JEOL JEM-1010 TEM.

#### **1.7 Dynamic light scattering (DLS) measurements**

DLS measurements (CP concentration 20 µM) were performed on a Malvern Zetasizer Nano ZSP. All measurements were done in triplicate and the average of the triplicate measurements was plotted.

#### **1.8 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)**

Precast gels 4-20% (Bio-Rad) were used for sample analysis. The samples were prepared with 4X non-reducing loading buffer (Bio-Rad) according to manufacturer's instructions. Gels were stained for proteins using Coomassie-Brilliant Blue (Bio-Rad). Images were acquired with a GE Image Quant 350 (GE healthcare Europa GmbH).

## 1.9 Agarose gel electrophoresis (AGE)

1% agarose gels were prepared in TEA buffer containing SYBR Gold (Thermo Fisher). Samples were mixed with gel loading dye and run for 40 minutes at 50 V. Gels were imaged before and after staining with Coomassie-Brilliant Blue (Bio-Rad) with a GE Image Quant 350 (GE healthcare Europa GmbH).

## 2 Experimental section

### 2.1 Cloning of CCMV variants

The pET-15b-G-H<sub>6</sub>-CCMV( $\Delta$ N26) as well as pET-15b-G-H<sub>6</sub>-ELP-CCMV( $\Delta$ N26) vectors encoding for the hexahistidine-tagged-CCMV proteins (V1 and V2) were previously constructed.<sup>1,2</sup> For the construction of variant 3 and 4 a set of DNA oligos, encoding for the entire ELP insert were designed and ordered (Table S2). These oligos were annealed at 95 °C and slowly cooled to room temperature. The resulting inserts encoded for a hexahistidine tag and 9 ELP repeats with sticky ends compatible with 5' NcoI and 3' XhoI annealing. The pET-15b vector was digested with NcoI and XhoI and the product was purified by agarose gel electrophoresis. Subsequently, the inserts were ligated into the digested vector. These plasmids were transformed into *E. coli* XL1-BLUE cells, the DNA was extracted and the sequences were confirmed by DNA sequencing (Table S2). For expression, the plasmids were transformed into *E. coli* BLR(DE3)pLysS cells (Novagen, MERCK).

Name	Sequence
V3-1	5'CATGGGCCATCATCATCATCACGTTCCGGGCGTCGGTGTTCCTGGACGTGGTGTTCAGGTGTAGGTGTTCAGGACTCGGTGTTCCTGGTGTAGGTGTTCCTGGTTAGGTGTTCAGGTGGCGGTGTTCCTGGTGGGAGTTCTCGGTTTAGGTC3'
V3-2	5'TCGAGACCTAAACCAGGAACCTCCACACCAGGAACACCGCCACCTGGAACACCTAAACCAGGAACACCTACACCAGGAACACCGAGTCCTGGAACACCTACACCTGGAACACCACTGGAACACCGAGCCCCGGAACGTGATGATGATGATGATGGCC3'
V4-1	5'CATGGGCACTCGTGCACAACGAAGGCATCATCATCATCACGTTCCGGGCGTCGGTGTTCCTGGATAGGTGTTCAGGTGTAGGTGTTCAGGACTCGGTGTTCCTGGTGTAGGTGTTCCTGGTTAGGTGTTCAGGTGGCGGTGTTCCTGGTGTGGGAGTTCTGGTTTAGGTC3'
V4-2	5'TCGAGACCTAAACCAGGAACCTCCACACCAGGAACACCGCCACCTGGAACACCTAAACCAGGAACACCTACACCAGGAACACCGAGTCCTGGAACACCTACACCTGGAACACCTAATCCAGGAACACCGAGCCCCGGAACGTGATGATGATGATGATGCCTTCGTTGTGCACGAGTGCC3'

**Table S2.** DNA sequence of oligos ordered for the construction of expression vectors.

Name	Sequence
Variant 1 Calculated MW (Da): 22506.6 Observed MW (Da): 22507	GSSHHHHHHSSGLVPRGSHMMSTVGTGKLTRAQRRRAAARKNKRNTRVVQPVIVEPI ASGQGKAIAWTGYSVSKWTASCAAAEAKVTSAITISLPNELSSERNKQLKVG RVLL WLGLLPSVSGTVKSCVTETQTAAASFQVALAVADNSKDVVAAMYPEAFKGITLEQ LTADLTIYLYSSAALTEGDVIVHLEVEHVRPTFDDSFPTVY
Variant 2 Calculated MW (Da): 22253.5 Observed MW (Da): 22252.7	GHHHHHHVPGVGPGLGVPVGPGLGVPVGPGLGVPVGGGVPVGPVGPGLGLEV VQPVIVEPIASGQGKAIAWTGYSVSKWTASCAAAEAKVTSAITISLPNELSSERNKQ LKVG RVLLWLGLLPSVSGTVKSCVTETQTAAASFQVALAVADNSKDVVAAMYPE AFKGITLEQLTADLTIYLYSSAALTEGDVIVHLEVEHVRPTFDDSFPTVY
Variant 3 Calculated MW (Da): 22296.5 Observed MW (Da): 22295.9	GHHHHHHVPGVGPGRGVPVGPVGPGLGVPVGPVGPGLGVPVGGGVPVGPVGPGLGLEV VQPVIVEPIASGQGKAIAWTGYSVSKWTASCAAAEAKVTSAITISLPNELSSERNKQ LKVG RVLLWLGLLPSVSGTVKSCVTETQTAAASFQVALAVADNSKDVVAAMYPE AFKGITLEQLTADLTIYLYSSAALTEGDVIVHLEVEHVRPTFDDSFPTVY
Variant 4 Calculated MW (Da): 23022.3 Observed MW (Da): 23021.6	GTRAQRRHHHHHHVPGVGPGLGVPVGPVGPGLGVPVGPVGPGLGVPVGGGVPVGPVGP GLGLEVVQPVIVEPIASGQGKAIAWTGYSVSKWTASCAAAEAKVTSAITISLPNELS SERNKQLKVG RVLLWLGLLPSVSGTVKSCVTETQTAAASFQVALAVADNSKDVVA AMYPEAFKGITLEQLTADLTIYLYSSAALTEGDVIVHLEVEHVRPTFDDSFPTVY

**Table S3.** Amino acid sequences and Q-TOF results of all proteins.

## 2.2 General protocol for the expression of His6-ELP-CCMV variants

CCMV variants were expressed according to a standard expression procedure.<sup>2,3</sup> *E. coli* BLR(DE3)pLysS cells containing pET-15b-CCMV vectors were inoculated overnight at 37 °C in LB medium (50 mL) containing Ampicillin (100 mg L<sup>-1</sup>) and Chloramphenicol (50 mg L<sup>-1</sup>). Subsequently, LB cultures were inoculated in 2YT medium (1 L) containing Ampicillin (100 mg L<sup>-1</sup>). Cultures were grown at 37 °C till an OD<sub>600</sub> of 0.5 was reached. IPTG (1 mM) was added and bacteria were grown at 30 °C for 6 hours. Cells were harvested by centrifugation at 2700 g for 15 minutes at 4 °C. Finally the cell pellet was stored overnight at -20°C. The cell pellet was resuspended in Lysis buffer (25 mL). Subsequently, cell lysate was obtained by ultrasonic disruption (3 times 30 seconds, 70 % amplitude, Branson Sonifier 150) and centrifuged at 16400 g for 15 minutes at 4 °C in order to remove cellular debris. The supernatant was incubated for one hour with Ni-NTA agarose beads (3 mL) at 4 °C. Finally, protein solutions were loaded onto a column. The flow-through was collected followed by two washing steps with Wash buffer. Proteins were eluted using the Elution buffer (1 time 0.5 mL, 7 times 1.5 mL). All fractions containing the CCMV protein were combined and dialyzed (12-14 kDa tubing) against dimer buffer pH 7.5 for 1.5 hours (buffer changed 3 times) and against capsid buffer overnight at 4 °C. Proteins were characterized by SDS-PAGE, SEC, Q-TOF, TEM and DLS.

### **2.3 Crosslinking of CCMV Nanoparticles.**

For a typical crosslinking procedure, ELP-CCMV capsids (V2) were first dialyzed in pH 5 buffer without EDTA (12-14 kDa MWCO, 3x 30 minutes at 4 °C). Protein samples (100 µM) were incubated with Ni<sup>2+</sup> for 30 minutes at 4 °C according to a 10:1 molar excess of Ni<sup>2+</sup> to capsid proteins. Protein solutions were dialyzed against PBS<sub>1</sub> (12-14 kDa MWCO, 3 times 30 minutes at 4°C) and incubated with DTSSP for one hour at room temperature to a final protein concentration of 50 µM. Finally, samples were dialyzed in PBS pH 7.2 (12-14 kDa MWCO, 3 times 30 minutes at 4 °C) and analyzed by DLS, SEC or TEM. siRNA or tRNA loaded CCMV nanoparticles (V1) were crosslinked according to the same procedure described above without the intermediate step involving Ni<sup>2+</sup> stabilization.

### **2.4 Cargo-Induced Assembly and Loading Efficiency**

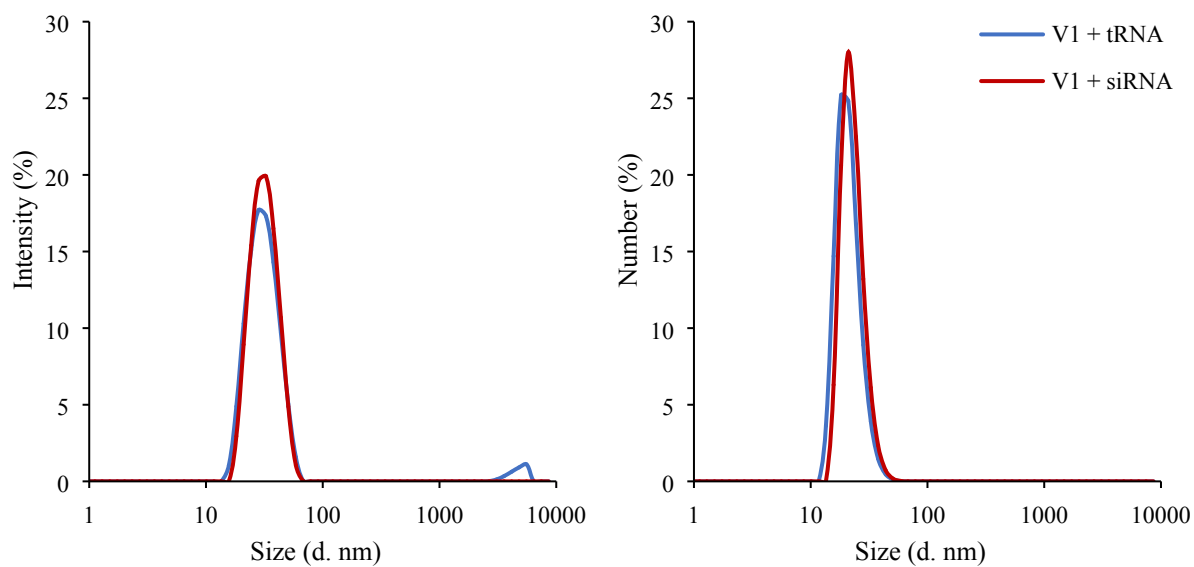
Protein solutions were dialyzed in dimer buffer pH 7.2 (12-14 kDa MWCO, 3x 30 minutes at 4 °C) and analyzed by DLS to assess particle disassembly. Capsid proteins were subsequently incubated with siRNA/tRNA (together with 10 eq of Ni<sup>2+</sup> for the ELP-CCMV variants V2, V3 and V4) for 30 minutes at 4 °C according to a 6:1 mass ratio of CP to oligonucleotides. The resulting virus-like particles were analyzed with DLS or TEM. For a standard evaluation of cargo loading agarose gel electrophoresis (running conditions: 1% agarose gel in 50 mM Tris buffer pH 7.2, 50V 40 minutes) together with UV-vis spectroscopy and Ribo green assay (according to manufacturer's instructions) were performed. Samples were analyzed immediately after siRNA/tRNA encapsulation as well as after dialysis in PBS and at the end of the crosslinking reaction.

### **2.5 Determination of the Efficacy of siRNA Encapsulation in Crosslinked Particles**

Prior to transfection, HeLa-Luc cells were grown on 96-well plates at 37 °C in a CO<sub>2</sub> incubator till 70% confluency. DMEM supplied with 10% (v/v) FBS and Penicillin-Streptomycin were used as culture medium. siRNA loaded crosslinked V1 nanoparticles (20 µM CP) as well as siRNA alone (0.068 g L<sup>-1</sup>) were incubated for 2 h at room temperature with Benzonase or PBS. Samples were diluted in Opti-MEM

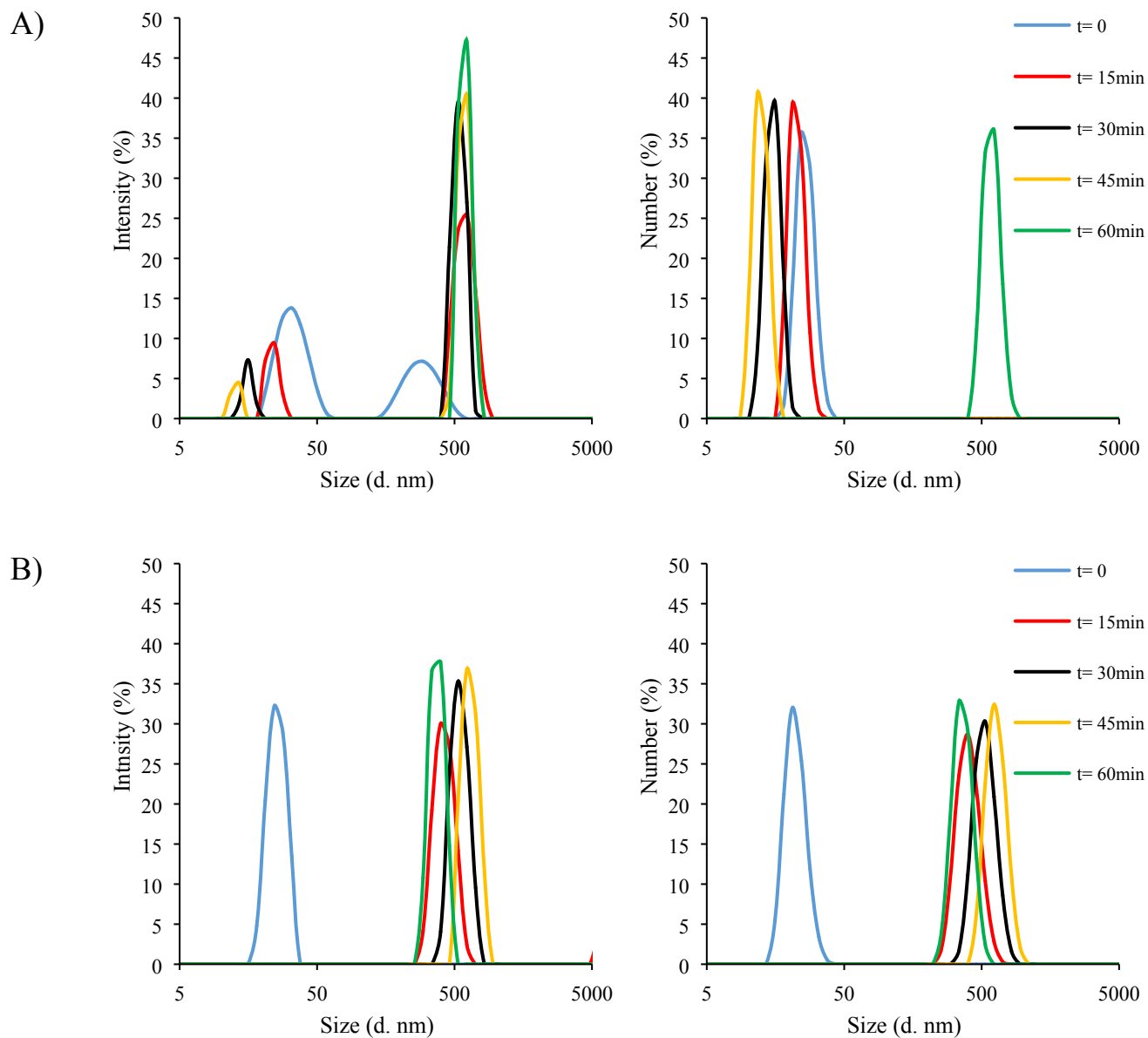
five times and incubated at room temperature for 5 minutes. A Lipofectamine solution (2 % v/v) was prepared in the same medium and incubated at room temperature for 5 minutes. Sample solutions and Lipofectamine solutions were mixed (1:1 v/v) and incubated at room temperature for 10 minutes. HeLa-Luc cells were washed twice with PBS and 100  $\mu$ L of Optimem were added per well. Finally, 50  $\mu$ L of the final solutions were used for the inoculation and cells were incubated at 37 °C. After 12 hours, solutions were replaced with fresh complete medium and cells were incubated at 37 °C for two days. Transfection efficacy was finally analyzed exploiting the Luciferase assay kit (Promega). Briefly, cells were washed twice with PBS before incubation with Lysis buffer (20  $\mu$ L per well). After 20 minutes the Luciferase assay reagent was added (100  $\mu$ L per well) and luminescence determined with a plate-reading Luminometer.

### 3 Supplemental figures

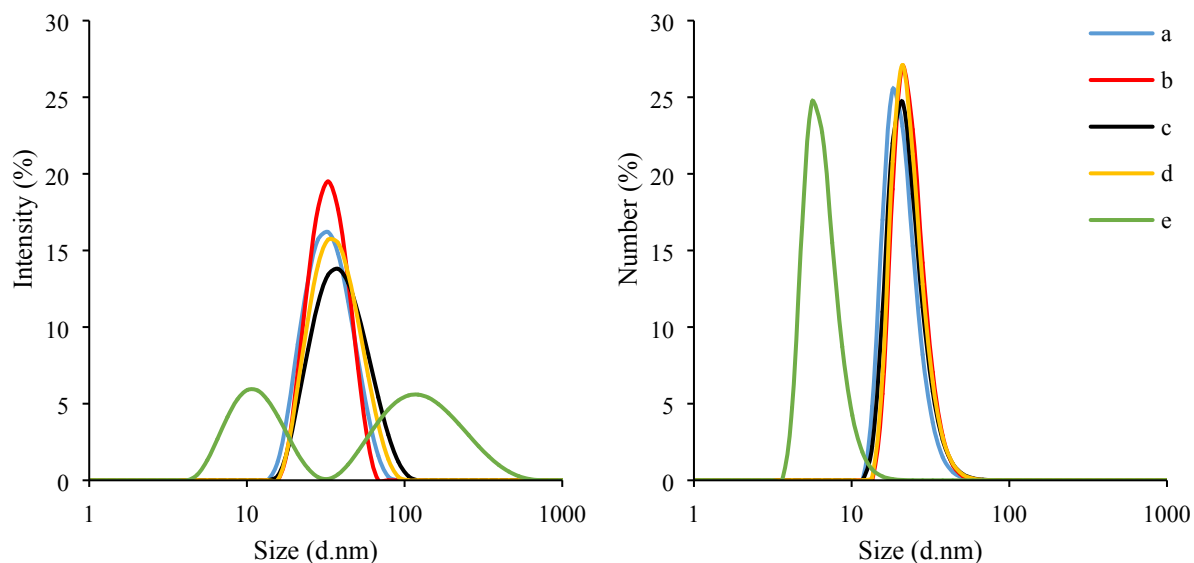


**Figure S1.** DLS analysis of V1 nanoparticles (20  $\mu$ M CP) after 4 hours incubation at 4  $^{\circ}$ C with tRNA and siRNA. Both Intensity (left) and Number (right) distribution plots show nanoparticles of 28 nm in diameter corresponding to assembled capsids.

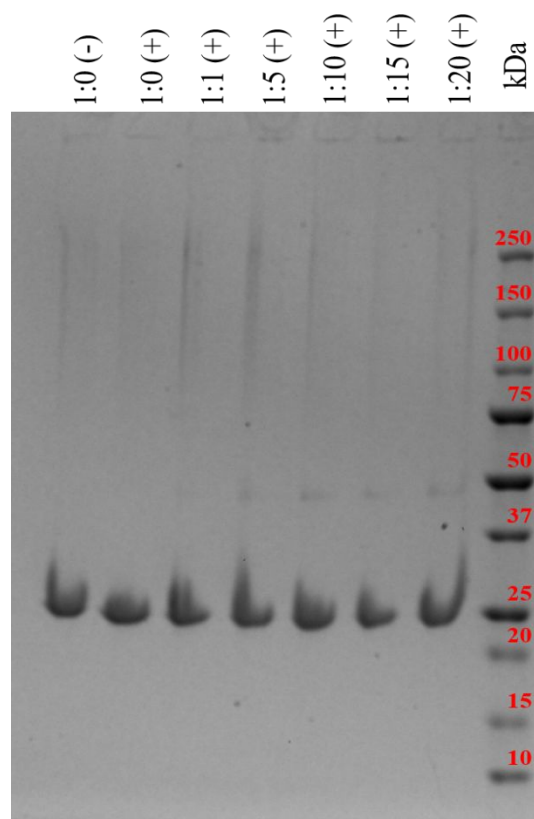




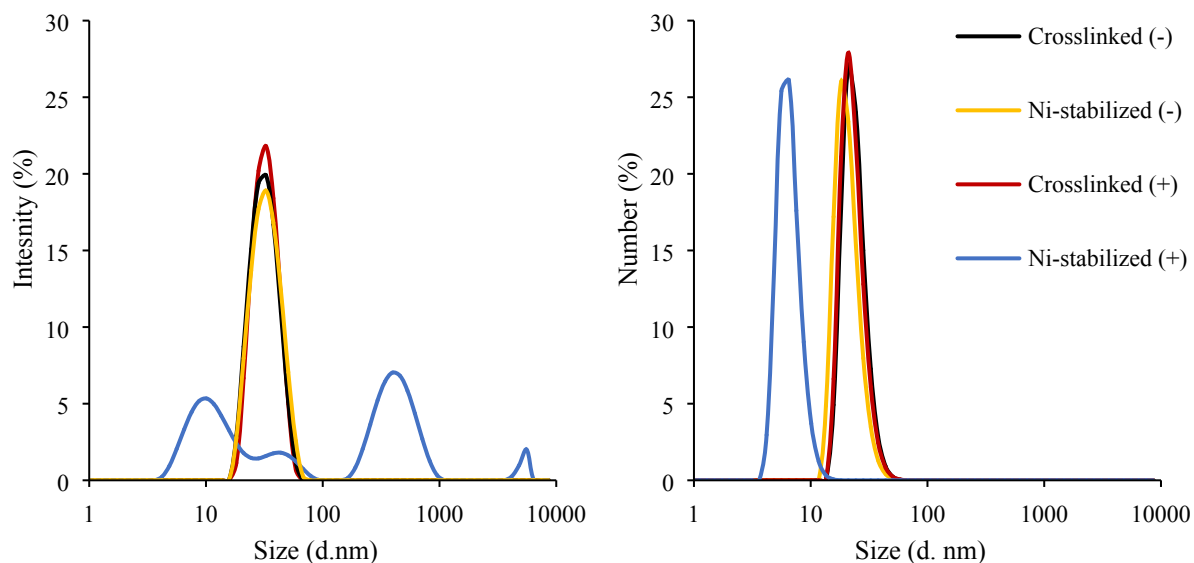
**Figure S2.** DLS analysis of V1 nanoparticles (20  $\mu$ M CP) loaded with siRNA (A) and tRNA (B) after incubation at 37  $^{\circ}$ C. Both Intensity (left) and Number (right) distribution plots show particle aggregation over time.



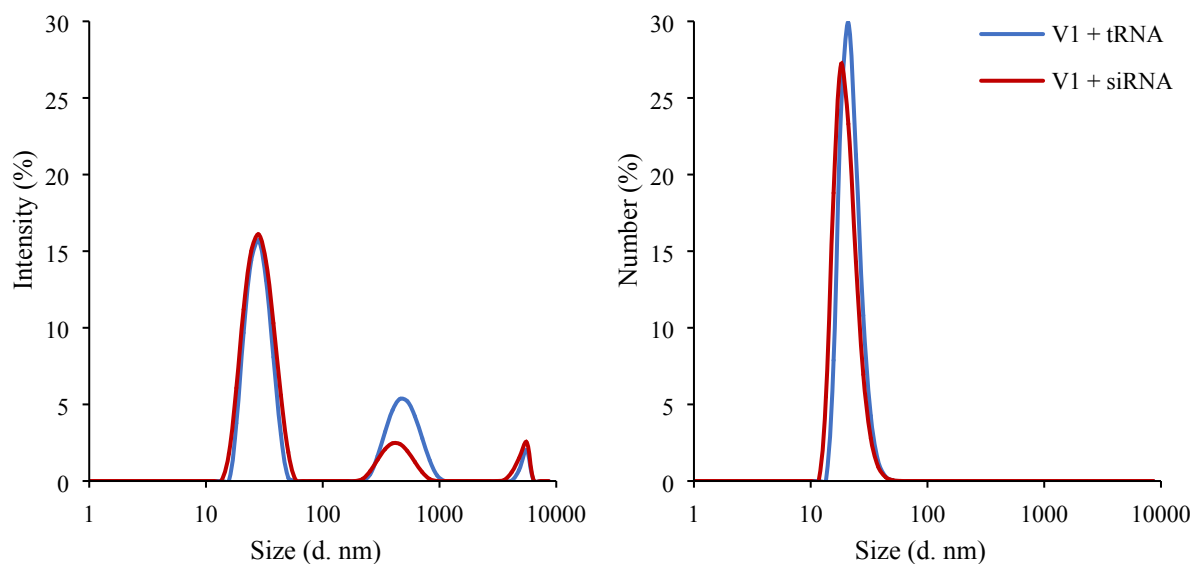
**Figure S3.** DLS analysis of V2 nanoparticles (20  $\mu$ M CP) before and after crosslinking with DTSSP (20:1 DTSSP: CP molar excess) as well as before and after addition of EDTA (2 mM) at 25  $^{\circ}$ C: a) V2 at pH 5 without EDTA, b) Ni-stabilized V2 at pH 7.2 without EDTA, c) Ni-stabilized crosslinked V2 at pH 7.2 without EDTA, d) Ni-stabilized crosslinked V2 at pH 7.2 with EDTA, e) Ni-stabilized V2 at pH 7.2 with EDTA. Both Intensity (left) and Number (right) distribution plots show stable capsids after addition of EDTA only in the presence of the crosslinker.



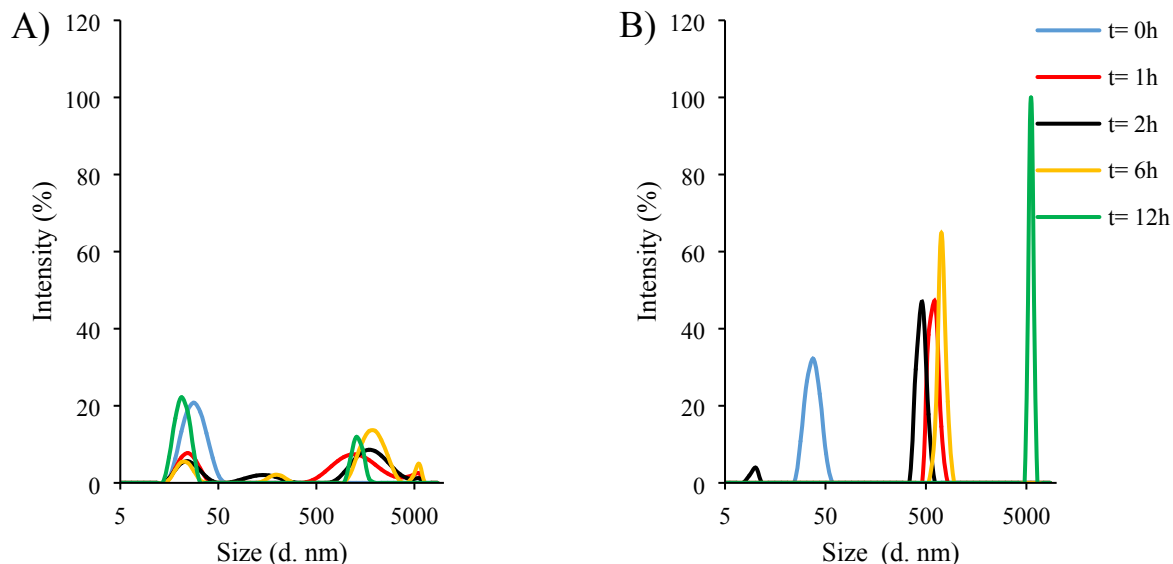
**Figure S4.** SDS-PAGE of V2 nanoparticles in PBS pH 7.2 after crosslinking with DTSSP at different concentrations (1:0, 1:1, 1:5, 1:10, 1:15, 1:20 CP: DTSSP molar ratio) after treatment with TCEP (50 mM). Nickel-stabilized particles with (+) and without (-) EDTA (2 mM) are used as negative and positive control respectively. Protein bands on gel electrophoresis were visualized with Coomassie staining. Bands associated to unmodified capsids as well as reduced crosslinked particles appear at approximately 22 kDa.



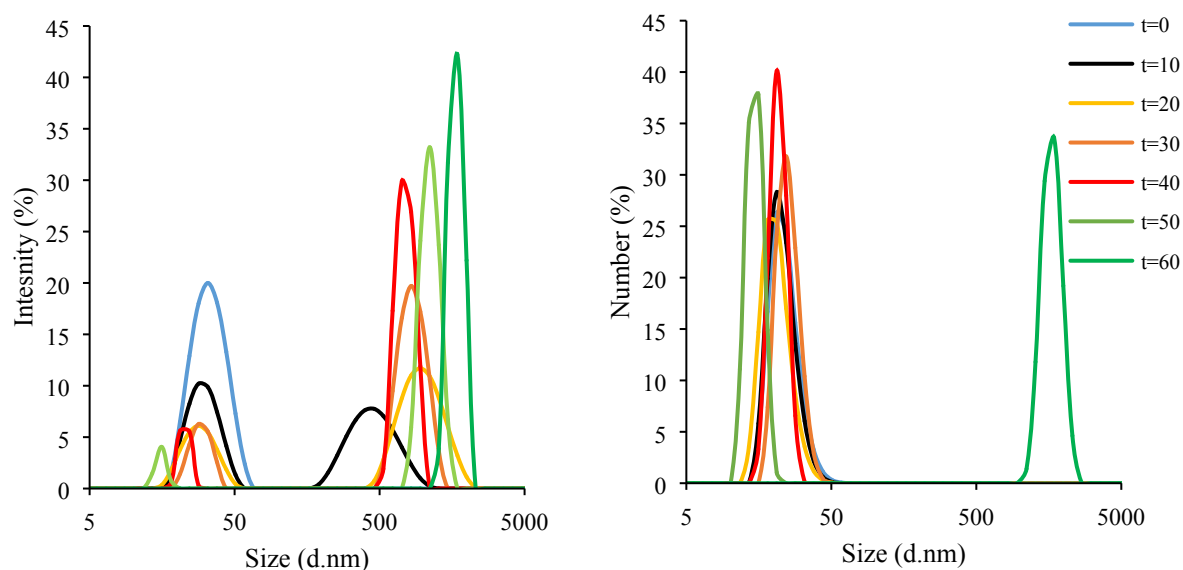
**Figure S5.** DLS analysis of V2 nanoparticles incubated in PBS at 25 °C for 6 hours (20  $\mu$ M CP) before and after crosslinking with DTSSP (20:1 DTSSP: CP molar excess). Both Intensity (left) and Number (right) distribution plots show the effect on particle stability over time in the presence (+) or absence (-) of EDTA (2 mM).



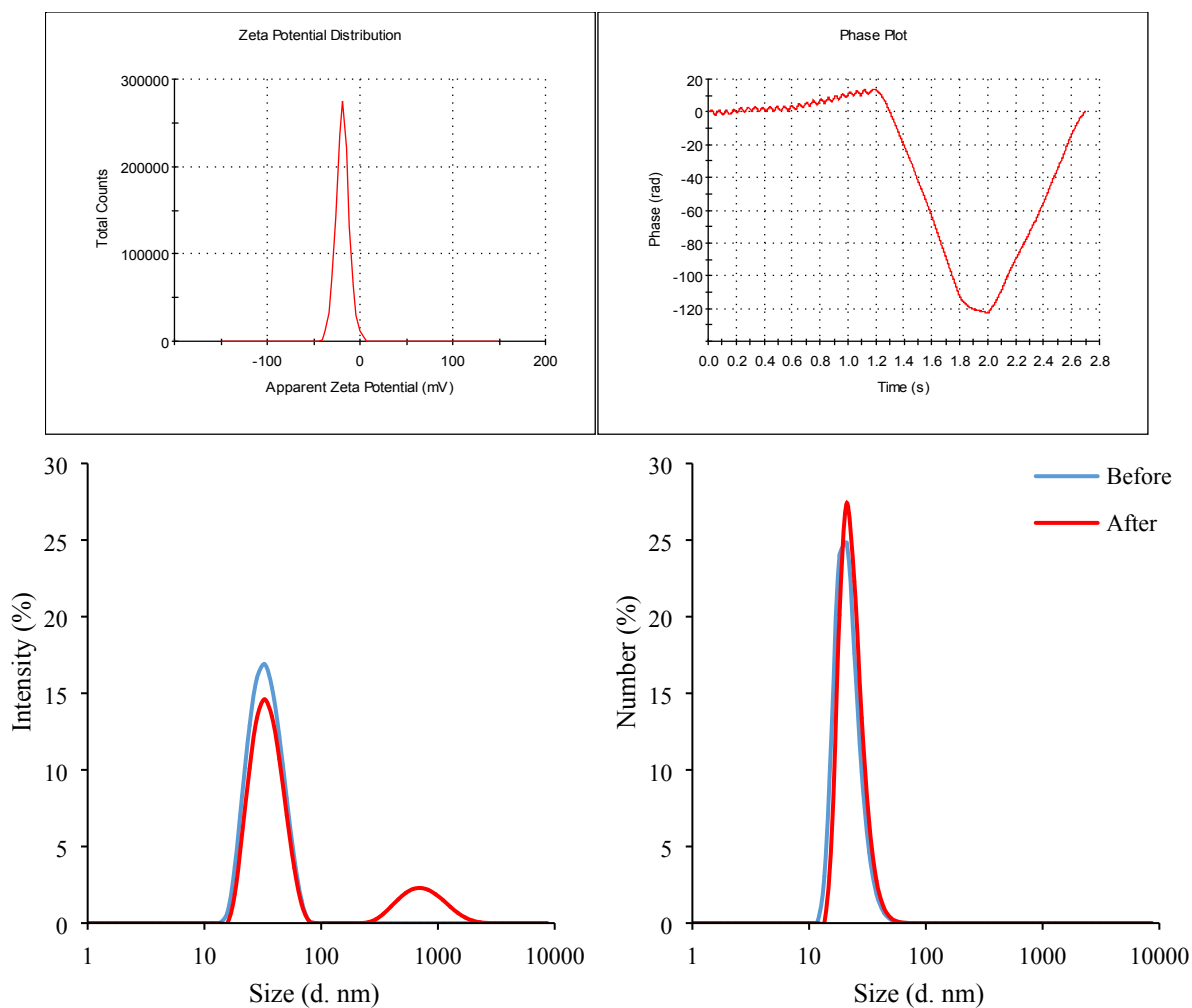
**Figure S6.** DLS analysis of crosslinked V1 nanoparticles in PBS (20  $\mu$ M CP) after tRNA and siRNA loading. Both Intensity (left) and Number (right) distribution plots indicate the presence of CCMV nanoparticles of 28 nm in diameter.



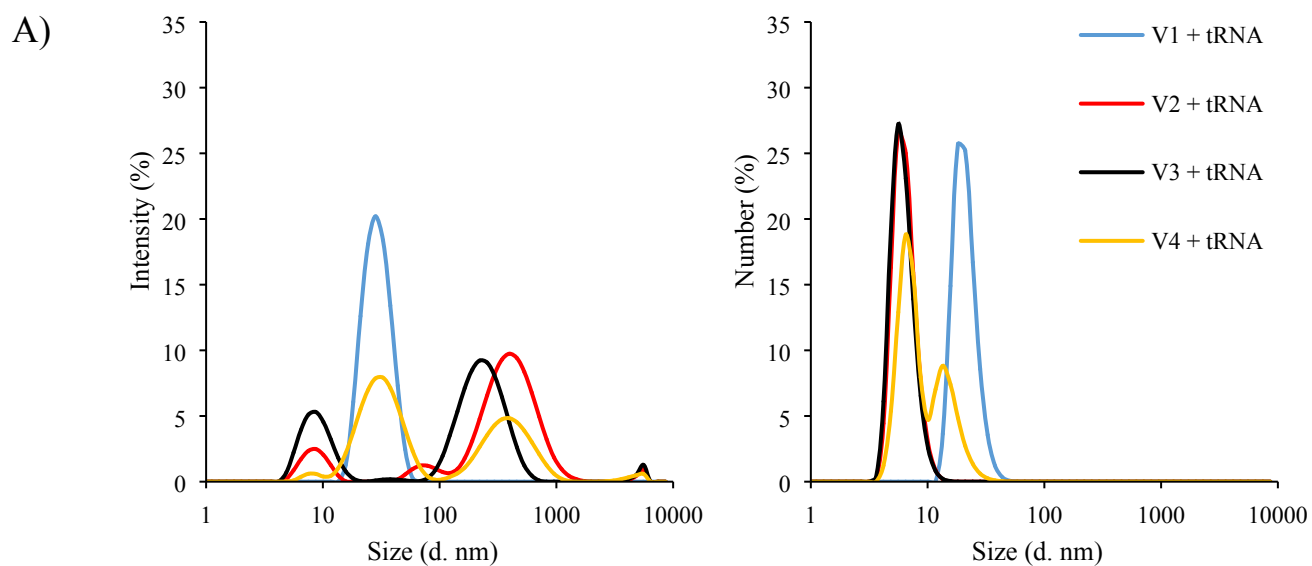
**Figure S7.** DLS analysis of CCMV nanoparticles in PBS after incubation at 37 °C for 0 (■), 1 (■), 2 (■), 6 (■) and 12 (■) hours: A) Ni-stabilized V2 capsid, B) siRNA-loaded V1 capsid. Intensity distribution plots show unstable particles and aggregation over time.



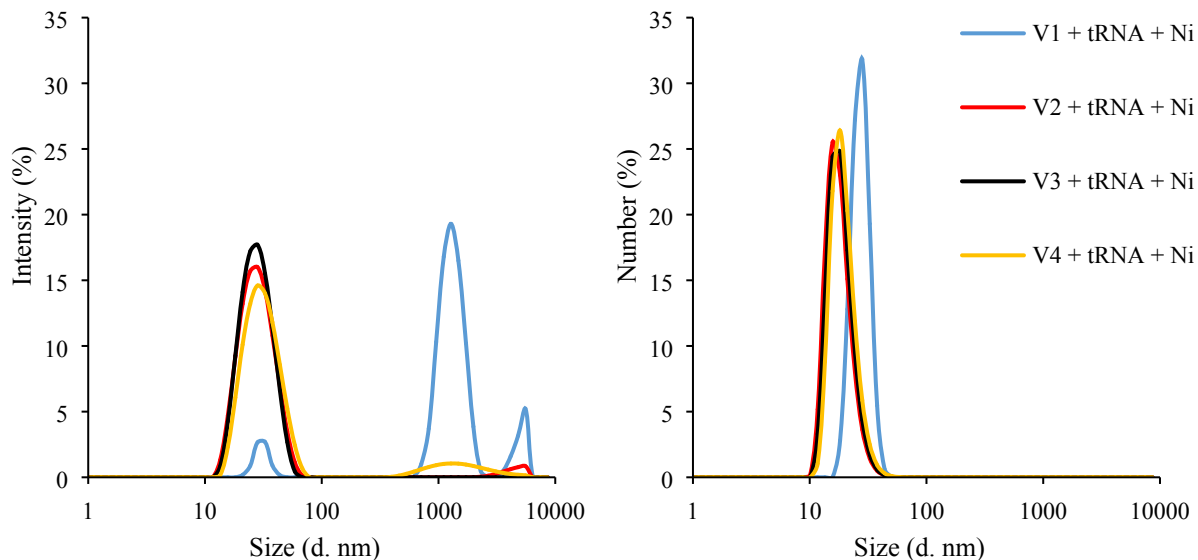
**Figure S8.** DLS analysis of crosslinked V2 nanoparticles in PBS (20  $\mu$ M CP) after treatment with 10 mM DTT at 37 °C every 10 minutes for one hour. Both Intensity (left) and Number (right) distribution plots indicate particle instability as a result of the reduction of the disulfide bond of DTSSP.



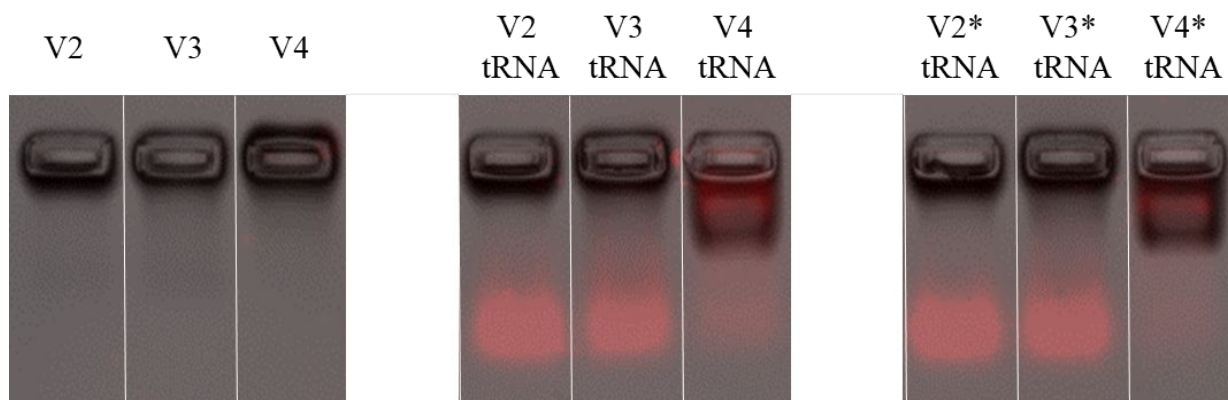
**Figure S9.** Zeta potential analysis (upper part) of crosslinked V2 nanoparticles (20  $\mu\text{M}$  CP) in water. Particle size was assessed before and after the zeta potential analysis (bottom part). Both the Intensity (left) and Number (right) distribution show stable particles.



B)

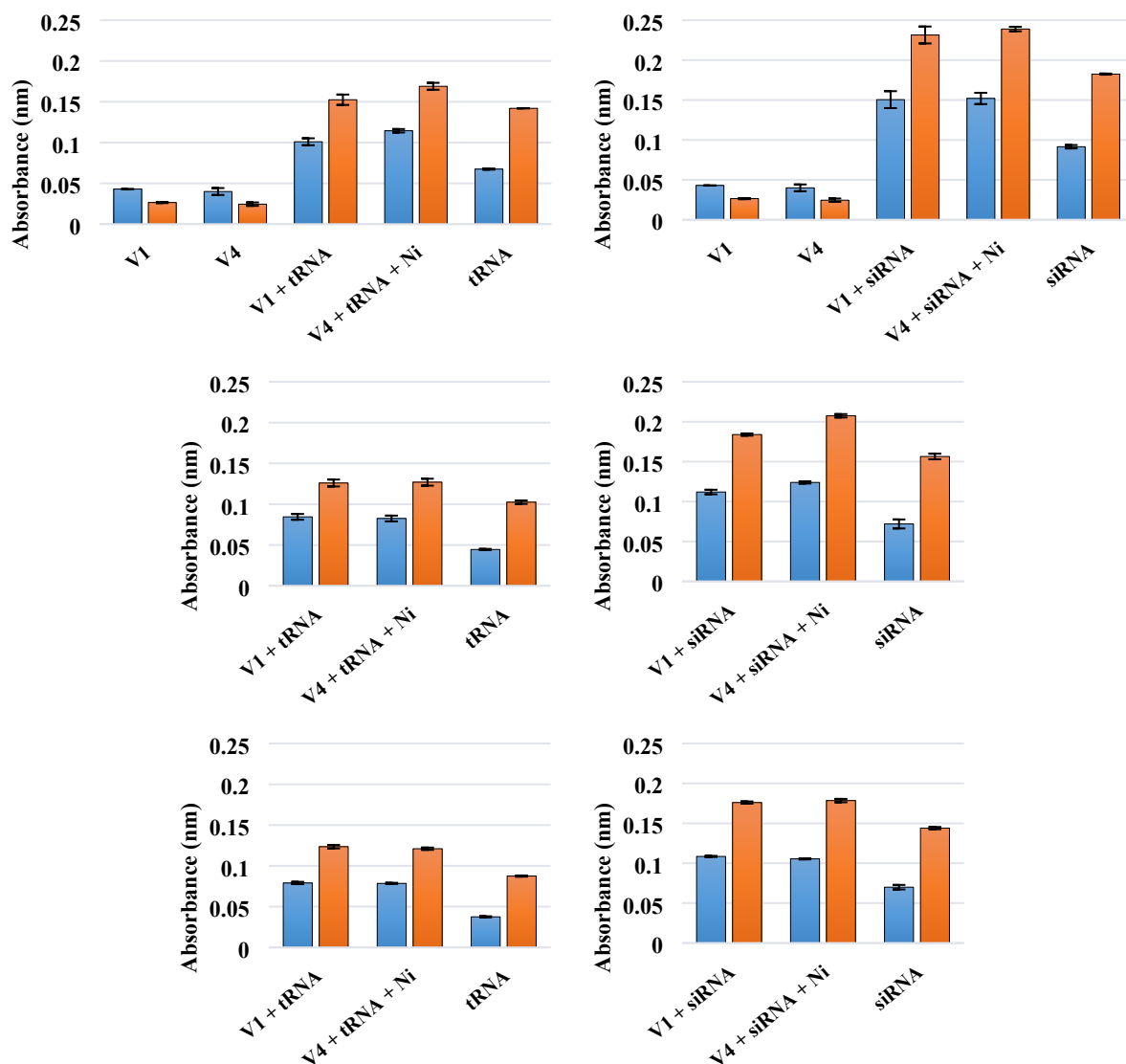


**Figure S10.** A) DLS analysis of CCMV variants (20  $\mu$ M CP) after incubation with tRNA (6.5:1 CP: tRNA mass ratio). Both the Intensity (left) and Number (right) distribution plots show stable particles with V1, no particles with V2 and V3 and a mixture of dimers and capsids with V4. B) DLS analysis of CCMV variants (20  $\mu$ M CP) after incubation with tRNA (6.5:1 CP: tRNA mass ratio) in the presence of  $\text{Ni}^{2+}$ . Both the Intensity (left) and Number (right) distribution plots show stable particles with V2, V3 and V4 and aggregates with V1.

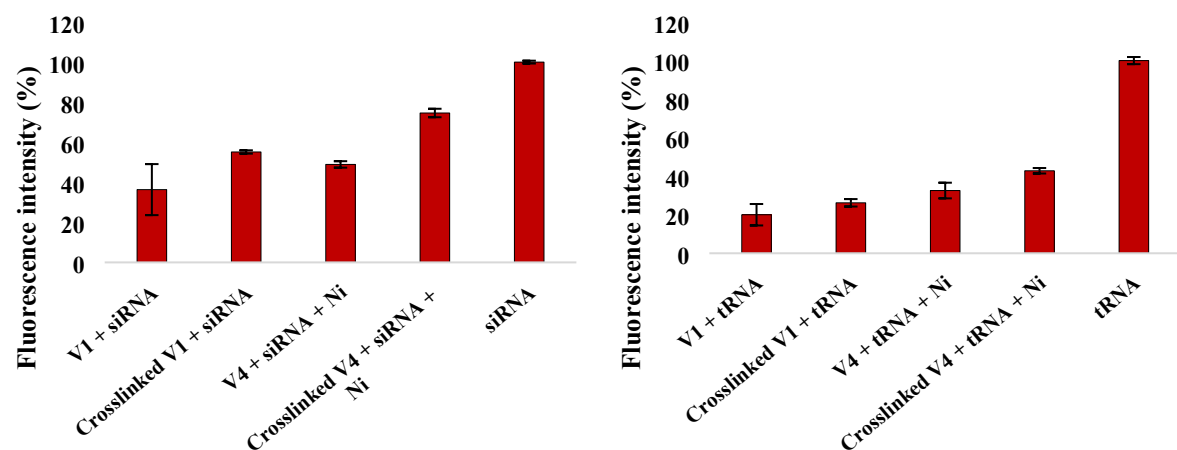


**Figure S11.** Agarose gel electrophoresis of V2, V3 and V4 dimers before and after incubation with tRNA, as well as in the presence of Ni (\*). Gels were stained with both Coomassie (grey) and SYBR Gold (red).

A)



B)



**Figure S12.** A) Uv-Vis analysis of samples loaded with siRNA (left) and tRNA (right) in assembly buffer (upper panel), after dialysis in PBS (middle panel) and after the crosslinking reaction with DTSSP (lower panel): ■) Abs<sub>260</sub>, ■) Abs<sub>280</sub>. Free siRNA and tRNA were used as controls. B) Evaluation of loading



efficiency exploiting Quant-iT RiboGreen RNA assay on V1 and V4 nanoparticles loaded with siRNA (left) and tRNA (right) after dialysis in PBS as well as after crosslinking. Free siRNA and tRNA were used as controls.

#### 4 References

- (1) Schoonen, L., Maas, R. J., Nolte, R. J., and van Hest, J. C. (2017). Expansion of the assembly of cowpea chlorotic mottle virus towards non-native and physiological conditions. *Tetrahedron* 73, 4968-4971.
- (2) van Eldijk, M. B., Wang, J. C. Y., Minten, I. J., Li, C., Zlotnick, A., Nolte, R. J., Cornelissen, J. J. L. M., and van Hest, J. C. (2012) Designing two self-assembly mechanisms into one viral capsid protein. *J. Am. Chem. Soc.* 134, 18506-18509.
- (3) Timmermans, S. B., Vervoort, D. F., Schoonen, L., Nolte, R. J., and van Hest, J. C. (2018) Self-Assembly and Stabilization of Hybrid Cowpea Chlorotic Mottle Virus Particles under Nearly Physiological Conditions. *Chem. - An Asian J.* 13, 3518-3525.