Supplementary Information

Delivery of siRNA therapeutics using cowpea chlorotic mottle virus-like particles

Patricia Lam,[a§] *Nicole F. Steinmetz*[a,b*]

[a.] *Department of Biomedical Engineering, Case Western Reserve University, Cleveland OH 44106.*
[b.] *Departments of NanoEngineering, Bioengineering, Radiology, Moores Cancer Center, University of California San Diego, La Jolla 92093.*
[*] *nsteinmetz@ucsd.edu*
[§] *Current address: Center for Gene Therapy, The Research Institute at Nationwide Children's Hospital, Columbus OH 43205*

Materials and Methods

Purification and propagation of cowpea chlorotic mottle virus (CCMV)

CCMV was propagated by mechanical inoculation using 5-10 μg of CCMV per leaf of cowpea plants, California Blackeye No. 5 (*Vigna unguiculata*). To isolate virus, infected leaf material was harvested 8 weeks post infection and blended with 2 mL of Buffer A (0.2 M sodium acetate buffer pH 4.8, 1 mM EDTA) per gram of tissue. The homogenate was squeezed through 3 layers of cheesecloth, collecting the liquid material. 1 volume of cold chloroform was added, mixed for 10 min and centrifuged at 15,000 x g for 15 min. The supernatant was collected and precipitated by adding NaCl to a final concentration of 0.02 M and 8% PEG8000. The mixture was stirred overnight at 4ºC, followed by centrifugation at 15,000 x g for 10 minutes. The supernatant was discarded and the pellet was resuspended in 20 ml Buffer B (0.1 M sodium acetate buffer pH 4.8, 1 mM EDTA) by stirring for 1h at 4ºC, then centrifuged at 8000 x g for 10 minutes. The supernatant was collected and centrifuged over a 20% sucrose cushion at 148,000 x g for 2 hours. The pellet containing purified virus was then resuspended in 1 ml Buffer B. The concentration of the CCMV was determined at A260 and $\varepsilon = 5.87\ \mu L\ \mu g^{-1}\ cm^{-1}$.

Disassembly CCMV particles to obtain coat proteins

To disassemble CCMV to get coat proteins, virions were dialyzed using a 3.5K MWCO Slide-a-Lyzer dialysis cassette (Thermo Scientific) in disassembly buffer (0.5 M $CaCl_2$, 50 mM Tris–HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF) at 4ºC for 24 hours. Following dialysis, the solution was centrifuged at 12,000 x g for 30 minutes at 4ºC to pellet the viral RNA. The supernatant was then centrifuged at 220,000 x g for 2 hours at 4ºC to pellet any non-disassociated virus particles. The supernatant containing coat proteins was then dialyzed using a 3.5K MWCO Slide-a-Lyzer dialysis cassette in protein buffer (1 M NaCl, 20 mM Tris pH 7.2, 1 mM EDTA, 1 mM DTT, 1 mM PMSF) for 24 hours and stored at 4ºC. The concentration of the CCMV coat proteins was determined at A280 and $\varepsilon = 1.27\ \mu l\ \mu g^{-1}\ cm^{-1}$.

Recombinant production and purification of CCMV coat proteins in *E. coli*

The CCMV coat protein (573bp) was cloned into the vector pET28a(+) (Novagen) via NdeI and BamHI. The pET281/CCMV-CP construct was transformed into the *E. coli* strain ClearColi BL21(DE3) (Lucigen). 2 mL of an overnight culture was transferred to 400 mL of LB-Miller broth with 50 mg L^{-1} kanamycin and grown at 37ºC until OD600 reached 0.6-0.8. Protein expression was induced using 0.5 mM IPTG and the culture was allowed to grow at 22ºC for 16 hours. The cultures were then placed on ice for 10 minutes and cells were harvested by centrifugation at 15,000 x g for 20 min at 4ºC. The supernatant was discarded and the pellet was resuspended in 20 mL bacteria lysis buffer (GoldBio) and incubated on ice for 5 minutes. Lysozyme was then added at a final concentration of 1 mg mL^{-1} and the cell suspension was incubated at 37ºC for 1 hour. PMSF was then added at a final concentration at 1 mM and the solution was sonicated on a Q500 Sonicator (QSonic) for 15 minutes using 5 second pulses at an intensity of 40%. The solution was centrifuged at 15,000 x g for 30 min at 4ºC and the supernatant was passed through a 0.45 μm filter. The cell suspension was loaded through a HisPur Cobalt Chromatography Cartridge (Thermo Scientific) and His-tagged CCMV coat proteins were collected through affinity purification as per manufacturer's protocol. 1.5 mL elutions were collected from the column using elution buffer (50 mM sodium phosphate, 300 mM sodium chloride, 250mM imidazole; pH 7.4). Elution fractions were measured using UV-visible spectroscopy to verify that proteins were present in the fractions, and fractions were pooled accordingly. Pooled fractions of CCMV coat proteins were then dialyzed in protein buffer (1 M NaCl, 20 mM Tris pH 7.2, 1 mM EDTA, 1 mM DTT, 1 mM PMSF) for 24 hours at 4ºC using a 3.5K MWCO Slide-a-Lyzer dialysis cassette (Thermo Scientific). Dialyzed coat proteins were stored at 4ºC.

Reassembly of CCMV

For reassembly, coat proteins subunits and the desired dicer substrate siRNA (IDT) were mixed in a 6:1 (w/w) ratio in protein buffer. The mixture was dialyzed in a 7K MWCO Slide-a-Lyzer dialysis cassette (Thermo Scientific) against RNA assembly buffer (50 mM Tris pH 7.2, 50 mM NaCl, 10 mM KCl, 5 mM $MgCl_2$, 1 mM DTT) for at least 6 hours at 4ºC, then immediately dialyzed against virus suspension buffer (50 mM sodium acetate buffer pH 4.5, 8 mM magnesium acetate) for at least 6 hours at 4ºC. The assembly was purified by centrifugation through a 100k Amicon Ultra-0.5 mL centrifugal filter (EMD Millipore) at 3000 x g for 5 minutes, followed by 3 washes with virus suspension buffer.

Transmission Electron Microscopy

CCMV samples were diluted to 0.5-0.8 mg ml^{-1} in water and 20 μL was applied to glow-discharged carbon-coated 200 mesh grids (Electron Microscopy Sciences) for 2 minutes. Excess sample was blotted from the grids with Whatman Grade 1 filter paper, and the grids were rinsed twice with distilled water before staining with 2% (w/v) uranyl acetate for 2 minutes. Grids were imaged on a FEI Tecnai Spirit T12 transmission electron microscope operated at 200 kV.

Chemical labelling of CCMV

Sulfo-Cy5 NHS ester (Lumiprobe) was conjugated to CCMV through NHS chemistry to the exterior surface lysines. The reaction was performed using a 100 molar excess of dye with CCMV in 0.1 M HEPES pH 7.0, 5 mM $MgCl_2$ buffer containing 10% (v/v) DMSO. The reaction was allowed to proceed overnight at room temperature with gentle agitation. The reaction was purified with ultracentrifugation at 150,000 x g for 1 hour over a 30% (w/v) sucrose cushion.

The peptide m-lycotoxin was conjugated to CCMV via an $SM(PEG)_4$ crosslinker (Thermo Scientific) through NHS chemistry to the exterior surface lysines. The reaction was performed using a 600 molar excess of $SM(PEG)_4$ with CCMV in 0.1 M HEPES pH 7.0, 5 mM $MgCl_2$ buffer for 2 hours at room temperature with gentle agitation. Excess $SM(PEG)_4$ was removed via centrifugation through a PD MidiTrap G-25 Sample Preparation Column (GE Healthcare). M-lycotoxin was reacted to CCMV-$SM(PEG)_4$ at 600 molar excess with gentle agitation over night at room temperature. The reaction was purified with ultracentrifugation at 150,000 x g for 1 hour over a 30% (w/v) sucrose cushion.

Gel Electrophoresis

For denaturing gel electrophoresis, samples were denatured by heating at 100ºC for 10 minutes in NuPage 4x LDS Sample loading buffer (Thermo Scientific). CCMV (10 μg) were loaded on 12% NuPage Bis-Tris protein gels (Thermo Scientific) and run in 1x MOPs buffer at 200V for 35 minutes. Gels were stained with Coomassie Blue. For native gel electrophoresis, 10 μg of sample was loaded into 0.8% (w/v) TAE agarose gels with 1X GelRed (Biotium) in 1x TAE buffer and run at 90V for 40 minutes. All gels were imaged on an AlphaImager HP (Protein Simple) and analyzed with Fiji.

Cell culture

HeLa and MCF-7 cells were obtained from the ATCC (Manassas, VA). HeLa/GFP cells were obtained from Cell Biolabs, Inc (San Diego, CA). Cells were grown and maintained in Dulbecco's Modified Eagle's medium (DMEM, Cellgro) supplemented with 10% (v/v) fetal bovine serum (Atlanta Biologicals) and 1% (v/v) penicillin/streptomycin (Gibco). Cells were grown at 37ºC in a 5% CO2 humidified incubator.

Flow cytometry

HeLa cells were collected using enzyme-free Hank's cell dissociation buffer (Gibco) and resuspended to 2.5 x 10^6 cells mL^{-1}. Cells (5 x 10^5 cells in 0.2 mL) were added to 96-well V-bottom plates (Corning 3897). CCMV particles (1 x 10^5 particles per cell) were added in triplicate and incubated for 6 hours at 37ºC in a 5% CO_2 humidified incubator. Following incubation, half of the cells were centrifuged at 500 x g and resuspended in DPBS with 1 mg mL^{-1} pronase (Sigma-Aldrich) and treated at room temperature for 15 minutes. All cells were then centrifuged at 500 x g and washed with FACS buffer (1 mM EDTA, 1% (v/v)

FBS, 25 mM HEPES pH 7.0 in PBS) twice and fixed in 2% (v/v) paraformaldehyde in FACS buffer for 10 minutes. After fixing, cells were further washed in FACS buffer twice. Following washing, cells were resuspended in PBS and analyzed on a BD LSRII instrument. At least 10,000 gated events were recorded and data were analyzed using FlowJo 10.2 software.

Confocal microscopy

HeLa or HeLa/GFP cells were seeded on circular coverslips in a 24 well suspension plate (25,000 cells in 0.5 mL). Cells were allowed to grow for 24 hours at 37ºC in a 5% CO_2 humidified incubator before 1 x 10^7 particles per cell were added. Following incubation with particles for 24 hours, cells were washed three times with DPBS, then fixed in 5% (v/v) paraformaldehyde, 0.3% (v/v) glutaraldehyde in DPBS for 10 minutes. Cells were then washed three times with DPBS. Cellular components were stained as follows: (A) for HeLa cells: (i) cell membranes were stained with wheat germ agglutinin, Alexa Fluor 555 conjugate (WGA-555; Invitrogen), 1:1000 in 5% (v/v) goat serum in DPBS; (ii) lysosomes were stained with an Alexa Fluor 488 anti-human LAMP-1 antibody (BioLegend), 1:500 in 5% (v/v) goat serum in DPBS); (iii) nuclei were stained with DAPI found in the mounting medium. (B) For HeLa/GFP cells: (i) cell membranes were stained with wheat germ agglutinin, Alexa Fluor 555 conjugate (WGA-555; Invitrogen), 1:1000 in 5% (v/v) goat serum in DPBS; (ii) nuclei were stained with DAPI found in the mounting medium; (iii) CCMV particles were stained with rabbit anti-CCMV antibody, 1:200 in 5% (v/v) goat serum in DPBS for 1 hour followed by goat anti-rabbit secondary antibody tagged with Alexa Fluor 647, 1:500 in 5% (v/v) goat serum in DPBS. Cells were first stained with WGA-555, then permeabilized with 0.2% (v/v) Triton X-100 for 2 minutes and then blocked with 10% (v/v) goat serum in DPBS for 1 hour. Cells were washed three times with DPBS in between treatments. Following all staining, coverslips were mounted onto slides with Fluoroshied with DAPI (Sigma-Aldrich) histology mounting medium and sealed with clear nail polish. Confocal images were obtained on a Leica TCS SPE confocal microscope with a 63x oil immersion objective. Images were analyzed with Fiji.

Cell treatment with siRNA and quantitative real-time PCR

HeLa/GFP or MCF-7 cells were plated in a 24-well treated plate at 62,500 cells mL^{-1}. 10,000,000 particles per cell of CCMV/CCM-siRNA was added to cells and incubated for 24 hours at 37°C in a 5% CO_2 humidified incubator. Treatments were performed in triplicate. For RNA extraction, cell media was removed and 0.5 mL of TRI-Reagent (Sigma-Aldrich) was added to the cells. Cell lysate was passed several times through a pipette to form a homogenous lysate. RNA was extracted using TRI-Reagent as per manufacturer's protocol. 1 µg of RNA was used to make cDNA using the iScript gDNA Clear cDNA synthesis kit (Bio-Rad). cDNA was diluted 1:10 and 2 µL was used in a 20 µL qPCR reaction containing 1x SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) and 250 nM each of forward and reverse primer (IDT). qPCR was performed using a CFX-96 touch machine (Bio-Rad) with the following parameters: 95°C for 30 seconds, then 40 cycles of 98°C for 10 seconds, 15 seconds at 60°C, followed by a melting curve. Data was analyzed with CFX Maestro software (Bio-Rad).

Statistical Analysis

Results are presented as means ± the standard deviation (SD). Statistical comparisons between groups were performed using a one-way ANOVA followed by the appropriate post hoc tests. Significance was accepted at p values <0.05.

dicer substrate siRNA		**sequence**
eGFP siRNA	sense	5’ AACGAGAAGCGCGAUCACAUGGUCC 3’
	anti-sense	5’ GGACCAUGUGAUCGCGCUUCUCGUUGG 3’
FOXA1 siRNA	sense	5’ GAGAGAAAAAAUCAACAGCAAACAA 3’
	anti-sense	5’ UUGUUUGCUGUUGAUUUUUUCUCUCUU 3’
primer		
eGFP	forward	5’ GAACCGCATCGAGCTGAA 3’
	reverse	5’ TGCTTGTCGGCCATGATATAG 3’
FOXA1	forward	5’ GGGGGTTTGTCTGGCATAGC 3’
	reverse	5’ GCACTGGGGGAAAGGTTGTG 3’
ACTB	forward	5’ AGGGTGAGGATGCCTCTCTT 3’
	reverse	5’ GGCATGGGTCAGAAGGATT 3’

Dicer substrate siRNA

eGFP siRNA

Sense 5’ AACGAGAAGCGCGAUCACAUGGUCC 3’
Antisense 5’ GGACCAUGUGAUCGCGCUUCUCGUUGG 3’

FOXA1 siRNA

Sense 5’ GAGAGAAAAAAUCAACAGCAAACAA 3’
Antisense 5’ UUGUUUGCUGUUGAUUUUUUCUCUCUU 3’

NEGATIVE CONTROL siRNA
IDT Negative Control DsiRNA NS1 (sequence not available)

Cy3-LABELED siRNA
eGFP-Cy3 siRNA (GE Dharmacon)

PRIMERS:
eGFP-F 5’ GAACCGCATCGAGCTGAA 3’
eGFP-R 5’ TGCTTGTCGGCCATGATATAG 3’
FOXA1-F 5’ GGGGGTTTGTCTGGCATAGC 3’
FOXA1-R 5’ GCACTGGGGGAAAGGTTGTG 3’
ACTB-F 5’ AGGGTGAGGATGCCTCTCTT 3’
ACTB-R 5’ GGCATGGGTCAGAAGGATT 3’

CCMV CP sequence:
ATGTCTACAGTCGGAACAGGGAAGTTAACTCGTGCACAACGAAGGGCTGCGGCCCGTAAG
AACAAGCGGAACACTCGTGTGGTCCAACCTGTTATTGTAGAACCCATCGCTTCAGGCCAAG
GCAAGGCTATTAAAGCATGGACCGGTTACAGCGTATCGAAGTGGACCGCCTCTTGTGCGGC
TGCCGAAGCTAAAGTAACCTCGGCTATAACTATCTCTCTCCCTAATGAGCTATCGTCCGAAA
GGAACAAGCAGCTCAAGGTAGGTAGAGTTTTATTATGGCTTGGGTTGCTTCCCAGTGTTAG
TGGCACAGTGAAATCCTGTGTTACAGAGACGCAGACTACTGCTGCTGCCTCCTTTCAGGTG
GCATTAGCTGTGGCCGACAACTCGAAAGATGTTGTCGCTGCTATGTACCCCGAGGCGTTTA
AGGGTATAACCCTTGAACAACTCGCCGCGGATTTAACGATCTACTTGTACAGCAGTGCGGC
TCTCACTGAGGGCGACGTCATCGTGCATTTGGAGGTTGAGCATGTCAGACCTACGTTTGAC
GACTCTTTCACTCCGGTGTAT**TAG**