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Cowpea Chlorotic Mottle Virus-Like Particles as Potential Platform for Antisense Oligonucleotide Delivery in Posterior Segment Ocular Diseases

Chiara Pretto, Miao Tang, Mei Chen, Heping Xu, Astrid Subrizi, Arto Urtti, and Jan C. M. van Hest*

Due to its small size, easy accessibility and immune privileged environment, the eye represents an ideal target for therapeutic nucleic acids in the treatment of posterior segment ocular diseases, such as age-related macular degeneration (AMD). Among nanocarriers that can be used to achieve nucleic acid delivery, virus-like particles (VLPs) obtained from the Cowpea chlorotic mottle virus (CCMV) are an appealing platform, because of their loading capacity, ease of manufacture and amenability for functionalization. Herein, antisense oligonucleotide-loaded CCMV nanoparticles, intended for intravitreal injection, are evaluated for selective silencing of miR-23, an important target in AMD. CCMV nanoparticles loaded with anti-miR-23 locked nucleic acid and stabilized using the 3,3′-dithiobis(sulfosuccinimidyl propionate) (DTSSP) cross-linker, were assembled in vitro with a loading efficiency up to 80%. VLPs are found to be stable at 37 °C in the vitreous humor up to 24 hours. Nanoparticle cytotoxicity, cellular uptake and transfection efficacy are evaluated in endothelial cells. Selective miRNA down-regulation is achieved by the loaded CCMV VLPs both in absence and presence of Lipofectamine, with efficacies of ≈40% and more than 80%, respectively. The authors’ findings pave the way for the future development of CCMV nanoparticles as oligonucleotide delivery platform to treat posterior segment ocular diseases.

1. Introduction

Age-related macular degeneration (AMD) is an ocular pathology affecting the posterior segment of the eye, in particular the central and cone-enriched area of the retina called macula, which is responsible for sharp vision. AMD causes vision impairment or vision loss and its worldwide spread represents a health care concern.[1] AMD is classified into a dry or nonexudative form and a wet or neovascular form.[2,3] Nonexudative AMD is associated with the abnormal formation of deposits of extracellular material called drusens at the interface between the Bruch’s membrane and the retinal pigment epithelium of the eye.[4] Even if dry AMD follows a slow course, it can cause in its final stage the disruption of the neurosensory retina (geographic atrophy) for which no cure is currently available. Wet AMD is associated with the onset of an angiogenic process originating from the choroid through the retina.[2,5] The formation of new blood vessels causes irreparable damage to the photoreceptors and leads subsequently to central vision loss. Patients affected by wet AMD are routinely treated with intravitreal injections of vascular endothelial growth factor inhibitors.[6] However, these treatments require a high frequency of administration and this entails low patient compliance as well as a burden for the healthcare system.[7,8] From this perspective, over the years, efforts were directed toward the discovery of new...
drugs and targets for this disease, but also the development of effective drug delivery systems that could improve targeting efficiency, stability, and duration of therapeutic action.

Due to its unique structure, the eye represents an optimal target for the delivery of nucleic acid-based drugs. It is easily accessible via several routes of administration, its small volume requires low drug doses and it has a relative immune-privileged environment, offered by the blood retinal barrier (BRB).[5–11] Nucleic acid-based pharmaceutics, including miRNA, siRNA, aptamers, and antisense oligonucleotides (ASOs), comprise a range of different, highly specific drugs that interfere with protein production. They allow selective degradation of specific endogenous RNA molecules or proteins responsible for several disorders, exploiting different mechanisms such as RNA interference, RNase H-mediated, or sterically mediated block of translation.[12,21] Among endogenous targets for the angiogenic process associated to wet AMD that can be addressed via therapeutic nucleic acids (TNAs), several miRNAs, also called angiomiRs, have been identified.[14–19] In particular, the miRNA family cluster 23–27 seems to play an important role, being upregulated in endothelial cells (ECs) and highly vascularized tissues.[20,21] This cluster is involved in the regulation of the expression of proteins such as Sprouty2, Semaphorin6A, and Semaphorin6D which have shown anti-angiogenic activity.[20,22]

The suppression of endogenous levels of miR-23 and miR-27 through anti-miR ASOs has proven to be effective in the suppression of angiogenesis in a laser-induced choroidal neovascularization mouse model.[16] As in this case, therapeutic nucleic acids (TNAs) under preclinical and clinical investigation are generally chemically modified in order to increase their stability (e.g., against endonucleases and exonucleases) and efficacy.[21–29] Most commonly used modifications include phosphorothioate oligos, 2′-O-methyl-modified oligos and locked nucleic acids (LNAs). For instance, in 1998 the first phosphorothioate antisense oligonucleotide Fomiviren (Vitravene) was approved for the treatment of cytomegalovirus retinitis and in 2004 Pegaptanib (Macugen) has been approved for the treatment of wet AMD. In this case, the phosphorothioate 2′-O-methylated and 2′-fluorinated oligonucleotide was conjugated to a 40 kDa PEG chain used to increase intravitreal residence time. Up to date, these are the only two TNA compounds commercially available for ophthalmological treatments. In addition to chemical modification, a more generic approach to improve TNA performance involves the use of cationic and/or lipophilic transfection reagents, which increase cell membrane penetration and cytosolic accumulation of oligonucleotides.[30–32] Furthermore, functionalized nanocarriers, modified with, for example, targeting agents and cell penetrating enhancers could increase their intracellular delivery and efficacy, thereby reducing dosages and potential immunogenicity.[33–37]

Viruses-like particles (VLPs) are an interesting class of nanocarriers for oligonucleotide delivery. They include the protein mantle of viruses without their genetic information. These protein particles are highly defined in composition and size and can be easily modified via protein engineering and chemical conjugation methods. Furthermore, as their natural function is the protection and delivery of nucleic acids they are optimally suited as transfection agents. Among the VLPs, protein cages obtained from the Cowpea chlorotic mottle virus (CCMV) represent an appealing nanoparticle platform. CCMV consists of 180 identical capsid proteins (CPs) organized to form a highly symmetrical icosahedral cage of 28 nm in diameter.[38] The capsid proteins can be conveniently produced using bacterial or yeast expression.[39–41] The most appealing feature of CCMV VLPs is their unique pH-responsive self-assembly behavior. Indeed, CCMV nanoparticles undergo disassembly at neutral pH and spontaneous assembly under acidic conditions (pH 5) even in the absence of the viral genome.[42,43] This reversible process can be exploited for the encapsulation of different cargos. In particular, thanks to the presence of positively charged residues at the N-terminus of the capsid protein, the addition of negatively charged polymers or oligonucleotides can induce electrostatic interactions that trigger nanoparticle assembly even at neutral pH.[44,45] This process leads to the formation of nanoparticles that resemble the native virus in terms of structure and morphology with the payload being protected in the inner particle cavity. In recent studies, CCMV VLPs have been used for the intracellular delivery of siRNA and other RNA molecules.[46–48] Moreover, we have previously shown that these protein cages can be further stabilized for nanomedical applications via a 3.3′-dithiobis(sulfosuccinimidyl propionate) (DTSSP)-mediated crosslinking of the protein shell after cargo-induced assembly.[48] This homobifunctional cross-linker, consisting of two amine-reactive N-hydroxysulfosuccinimide (sulfo-NHS) ester groups linked by an 8-carbon spacer, has been selected because it contains a disulfide bond within the structure that can be reduced in a reducing environment within the cell cytosol. Therefore, DTSSP-mediated stabilization of nanoparticles is reversible and should enable cargo release after cellular uptake. In the present work, CCMV VLPs, loaded with antisense nucleotides, which is therapeutically relevant for the treatment of AMD, were investigated in vitro. First, LNA-loaded nanoparticles were generated and characterized after DTSSP-mediated stabilization. For the first time, LNA-loaded CCMV VLP stability was assessed in the vitreous humor extracted from porcine eyes. The cytotoxicity and uptake of the nanoparticles was subsequently tested in mouse brain endothelial cells (bEnd.3 cells), selected as a cell model used in vasculature research. Last, the efficacy of the nanoparticles, in the presence or absence of Lipopectamine, was investigated. This research represents the first step toward the development of CCMV VLPs for intravitreal delivery of therapeutic oligonucleotides to the posterior segment of the eye.

2. Results and Discussion

2.1. LNA-Induced Nanoparticle Assembly and Characterization

CCMV nanoparticles loaded with LNA (Table S3 and Figure S1, Supporting Information) were generated at neutral pH (encapsulation buffer, Table S1, Supporting Information) by mixing CCMV CP dimers with oligonucleotides according to a CP:LNA mass ratio of 6.5:1 leading to a final LNA concentration of 68 µg mL⁻¹. After 24 h of incubation, the generated capsids were cross-linked in PBS using DTSSP in order to further stabilize the particles. Capsid proteins (CPs) incubated with only water were used as the negative control since at neutral pH and in the absence of a negatively charged payload, CPs are only present in their dimeric state. VLP assembly and nanoparticle size were determined
using DLS (Figure 1A) and proved to be similar to the empty particles formed under acidic conditions (Figure S2B, Supporting Information). As expected, no particle formation was observed in the absence of the oligonucleotides (CCMV CP dimers in Figure 1A). Gratifyingly, the ribose modification of the LNA backbone did not affect the oligonucleotide-CP electrostatic interactions responsible for nanoparticle formation at neutral pH. Moreover, the short (15-oligonucleotides long) and chemically modified sequences of oligonucleotides were shown to be able to induce full capsid assembly within 24 h. This process was shown to be faster compared to previously reported native CCMV capsids obtained using oligonucleotides of similar length. [49] Cross-linked CCMV nanoparticles loaded with LNA scramble and LNA anti-miR-23a/b showed an average diameter of 30.2 nm (SD = 0.4, PDI = 0.284) and 31.2 nm (SD = 0.4, PDI = 0.249), respectively. The homobifunctional cross-linker was used to covalently link amino groups of different CPs on the particles surface. SDS-PAGE analysis depicted in Figure 1B was performed to assess successful cross-linking of CCMV nanoparticles after cargo loading. Under the experimental conditions used (50 mM DTT), nanoparticles are not fully reduced in solution. Indeed, the expected band of the CPs at about 25 kDa was observed, along with higher molecular weight structures and bands above 250 kDa corresponding to cross-linked capsids. No higher ordered structures were observed in CCMV CP dimers or LNA-loaded CCMV capsids before cross-linking used as negative controls (Figure S3, Supporting Information). Last, the expected spherical morphology of cross-linked LNA-loaded CCMVs and the homogeneity of the formulation were further confirmed by TEM analysis (Figure 1C). The average nanoparticle size (mean = 26.4 nm SD = 2.4) was in agreement with DLS data as well as with the size of empty CCMV VLPs in acidic conditions (mean = 30.3 nm SD = 2.2) (Figure S2D, Supporting Information).

2.2. Assessment of LNA Encapsulation and Loading Efficiency

LNA encapsulation into CCMV nanoparticles was verified using agarose gel electrophoresis as depicted in Figure 2A. Since free and encapsulated oligonucleotides have different electrophoretic mobility in the gel, SYBRGold staining was performed to visualize both species in solution while Coomassie staining was used to localize the protein components. The overlap between protein staining and oligonucleotide staining proved the successful encapsulation of the cargo into the protein cages. No discrepancy was observed between cross-linked nanoparticles loaded with LNA anti-miR-23a/b and the scrambled control, indicating that the differences in the nucleotide sequences have no effect on the final assembly. Moreover, lower-order structures or LNA smears were not observed indicating again the high homogeneity of the formulations. As a further proof, nanoparticles were characterized using SEC (Figure 2B) and the absorbance spectra at 260 and 280 nm were recorded. These wavelengths were selected to simultaneously visualize CCMV VLPs (aromatic amino acids have a maximum absorption at 280 nm) and the oligonucleotides (nucleobases strongly absorb at 260 nm). Empty CCMV nanoparticles were identified at an elution volume of about 11 mL, where the absorbance at 280 nm is higher than the absorbance at 260 nm (Figure S4, Supporting Information). As expected, CCMV nanoparticles loaded with LNA scramble and LNA anti-miR-23a/b also eluted at 11 mL indicating complete capsid assembly. Moreover, the 260 nm signal was higher compared to the 280 nm, indicating successful LNA encapsulation into the VLPs. Residual free LNA after encapsulation eluted at a later time point (about 18 mL). The encapsulation efficiency of nanoparticles before and after cross-linking was investigated exploiting the RiboGreen assay (Figure 3). All samples were buffer exchanged using a dialysis membrane with a cut-off size of 3.5 kDa in order to
prevent free oligonucleotide removal. CCMV nanoparticles loaded with LNA scramble and LNA anti-miR-23a/b showed a loading efficiency of 71% (SD = 3) and 87% (SD = 4) respectively. This variation may be a result of the differences in the oligonucleotide sequences and their flexibility in solution, thus affecting their interaction with the CPs. After cross-linking the loading efficiency dropped to 59% (SD = 4) and 78% (SD = 1) respectively. This was expected, since cargo leakage has been shown before in siRNA-loaded CCMV nanoparticles and may be explained by a partial displacement of oligonucleotides electrostatically bound to the external surface of the particles, as a consequence of the cross-linking reaction.\(^{[36]}\)

### 2.3. Stability of LNA-Loaded Nanoparticles under Physiological Conditions

In order to evaluate the applicability of LNA-loaded nanoparticles in ophthalmological treatments and in particular intravitreal injections, the stability of samples was assessed at 37 °C in PBS and ex vivo in the vitreous humor of porcine eyes. The stability of nanoparticles with and without cross-linking was followed for 72 h (Figure 4, Figure S5, Supporting Information). Sample analysis was performed exploiting native gel electrophoresis, where SYBRGold staining was used to localize encapsulated and free oligonucleotides, while Coomassie staining was performed to visualize the proteins of LNA-loaded nanoparticles. PBS, CCMV CP dimers and free LNA scramble were used as negative controls. Without cross-linking, nanoparticles (sample 3 in Figure 4) showed a fast degradation in both PBS and vitreous. This is attributed to their lower thermal stability in the absence of the cross-linker resulting in protein aggregation. Indeed, both protein and LNA bands disappeared within 3 h of incubation. On the contrary, cross-linked nanoparticles (sample 4 in Figure 4) were stable up to 24 h in PBS and residual VLP signal was present even after 3 days (Figure S5, Supporting Information). In the vitreous humor, cross-linked nanoparticles were stable up to 12 h. Nanoparticles were still visible after 24 h of incubation. The
2.4. Cell Uptake and Cytotoxicity

Next, the cytotoxicity of LNA-loaded nanoparticles was investigated in bEnd.3 cells exploiting the alamarBlue assay. This cell line was selected as it represents a relevant model in vasculature research and it can therefore be used to investigate exudative AMD. Prior to cytotoxicity evaluation, the cells were incubated with LNAs-CCMV-DTSSP nanoparticles and LNA23a/b-CCMV-DTSSP nanoparticles in the presence of Lipofectamine 2000 at different concentrations (0.5–2 µm final CP concentration). In this way we could identify the optimal conditions to be used for further transfection experiments. Untreated cells as well as cells incubated with Lipofectamine-only were used as negative controls. Results are displayed in Figure 5A. Moderate cytotoxicity was observed at the highest concentrations tested (1 and 2 µm CP). This was observed for both nanoparticles regardless of the LNA sequence. Consequently, further in vitro experiments were performed using a final CP concentration of 0.25 µm. We further analyzed the uptake in the same cell line using CCMV nanoparticles chemically functionalized with a green fluorescent dye via NHS coupling. As depicted in Figure 5B, cell internalization was observed for LNAs-CCMV-DTSSP and LNA23a/b-CCMV-DTSSP nanoparticles 12 h post incubation, even in the absence of the transfection reagent.

2.5. Knockdown Efficacy

The transfection efficiency of free LNA and LNA-loaded CCMV nanoparticles was assessed in bEnd.3 cells via RT-qPCR analysis of the miR-23a expression level. This experiment was performed in order to verify: i) the specificity of LNA anti-miR-23a/b for the target miRNA compared to the scramble control; ii) the effect of CCMV nanoparticles on LNA cytosolic release in the presence of Lipofectamine; and iii) the efficacy of LNA-loaded CCMV nanoparticles on the expression of miRNA23a in the absence of the transfection agent. Scrambled LNA sequence was selected as a negative control since it should have no effect on the regulation of the miR-23a expression level. Expression changes, normalized to the control (untreated sample), are reported in Figure 6. As expected, LNA anti-miR-23a/b, in the presence of Lipofectamine (L), showed a high knockdown efficacy (>80%) in bEnd.3 cells and high specificity for miR-23a compared to scramble LNA. Moreover, CCMV nanoparticles, loaded with LNA anti-miR-23a/b and the scramble control, coincubated with Lipofectamine, showed comparable suppression of miRNA23a expression indicating that the nanoparticle structure and the presence of the cross-linker are neither affecting LNA release nor compromising LNA efficacy in vitro. Furthermore, CCMV nanoparticles, even with a lower efficiency (∼40%), were also shown to be able to decrease the expression of miR-23a in the absence of the transfection agent. This lower efficacy may be attributed to CCMV nanoparticles’ endosomal accumulation after cellular uptake, which has already been reported in different cell lines.\(^\text{(50)}\) Since CCMV VLPs used in our studies were not functionalized on the particle surface with targeting agents or fusogenic moieties, Lipofectamine had to be used to maximize the transfection efficiency by inducing endosomal escape. However, from our data it can be deduced that the functionalization of CCMV VLPs with the above-mentioned functional groups could make their effectiveness comparable to the combination treatment with transfection reagent.

3. Conclusions

In the present work, we reported a versatile, reproducible, and fast approach to generate CCMV VLPs loaded with short antisense oligonucleotides targeting one member of the miR-23–27–24 cluster family involved in the development of AMD. Chemical modifications of ASOs did not hinder nanoparticle assembly in vitro and the resulting VLPs were fully characterized in terms of size, morphology, cargo encapsulation, and loading efficiency. Moreover, DTSSP-mediated cross-linking of VLPs yielded stabilized nanoparticles in vitreous humor. The safe concentrations of the nanoparticles and their cellular uptake were evaluated. Finally, the transfection efficacy of the nanoparticles was evaluated via RT-qPCR analysis showing a high specificity of LNA anti-miR-23a/b for the target miRNA23a compared to the scramble control as well as the possibility to induce a moderate knockdown effect, even in the absence of the transfection reagent. These results pave the way for the development of
Figure 4. Native gel electrophoresis showing the stability of CCMV nanoparticles loaded with LNA scramble. The analysis was performed in PBS and vitreous humor after incubation at 37 °C over time. PBS (1), CCMV CP dimers (2), LNAs-CCMV (3), LNAs-CCMV-DTSSP (4), LNAs (5). SYBRGold staining and Coomassie staining were performed to visualize oligonucleotides and proteins, respectively. The black arrow indicates the position of LNA-loaded nanoparticles while the red arrow indicates free LNA in solution.

Figure 5. A) Evaluation of CCMV nanoparticle cytotoxicity performed on bEnd.3 cells exploiting the alamarBlue assay. Cell viability was normalized to untreated cells. Mean values, standard deviations (n = 4) and selected statistical analysis of samples compared to untreated cells (ns: not significant; ****p < 0.0001) are reported. B) Qualitative analysis of nanoparticle uptake in bEnd.3 cells after 12 h of incubation in the absence of Lipofectamine. Nuclei are stained in blue, CCMVs are stained in green and actin filaments in red. The scale bar is 100 µm.
Figure 6. Evaluation of the transfection efficiency of free and encapsulated oligonucleotides in bEnd.3 cells. mRNA23a expression fold change values were normalized to untreated cells (controls). Samples coincubated with lipofectamine 2000 are indicated with (L). Mean values, standard errors (n = 3), and statistical analysis of samples compared to the controls (ns: not significant; *p < 0.05; **p < 0.01) are reported.

4. Experimental Section

Materials: DTSSP (Cat. No. 21 578), DyLight 488-NHS (Cat. No. A20000), Alexa Fluor 594 Phalloidin (Cat. No. A12381), TaqMan MicroRNA Assays (hsa-miR-23a-3p, Cat. No. 4 427 975), TaqMan Fast Advanced Master Mix (Cat. No. 4 444 558), TaqMan MicroRNA Reverse Transcription Kit (Cat. No. 4 366 596), the alamarBlue reagent (Cat. No. DAL1025), SYBR-Gold (Cat. No. S11494), and lipofectamine 2000 (Cat. No. 1 668 019) were purchased from ThermoFisher Scientific (Waltham, Massachusetts, USA). Valiated TaqMan probe β-actin (Cat. No. 307 903) was purchased from Roche (Basel, Switzerland). Bovine serum albumin (Cat. No. A3803) and DAPI (Cat. No. D9542) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Quant-IT RiboGreen RNA Reagent was obtained from Invitrogen (Carlsbad, California, USA). Oligonucleotides were purchased from Qiagen (Hilden, Germany), sequences can be found in the Supporting Information. CCMV VLPS were expressed in E. coli and fully characterized as described in the Supporting Information.

UV-Vis Absorbance Measurements: The concentration of capsid proteins (22 507 g mol⁻¹) as well as LNA scramble (4875.91 g mol⁻¹) and LNA anti-miR-23a/b (4946.95 g mol⁻¹) was calculated from the absorbance at 280 and 260 nm respectively, exploiting the theoretical extinction coefficients (23 470, 143 100, and 140 900 L mol⁻¹ cm⁻¹, respectively) using a spectrophotometer ND-1000 (Pregab Biotechnologie GmbH).

Preparation of Cross-Linked LNA-Loaded CCMV Nanoparticles: CCMV VLPS were first dialyzed (12–14 kDa Spectra/Por dialysis tubing, Spectrum) in encapsulation buffer (Tris buffer, pH 7.2, Table S1, Supporting Information) for 90 min at 4 °C (buffer changed three times) using a start-up CP concentration of 50 µm. After dialysis, samples were centrifuged twice at 12 100 g for 1 min and the protein concentration was measured. Last, particles were incubated with a 10-molar excess of DTSSP for 1 h at 600 rpm at 21 °C using an Eppendorf thermomixer (ThermoFisher Scientific) followed by extensive overnight dialysis in PBS at 4 °C to remove the unreacted reagent in the solution.

Preparation of Labeled Cross-Linked LNA-Loaded CCMV Nanoparticles: Cross-linked and LNA-loaded nanoparticles were diluted in PBS to a final CP concentration of 20 µm and directly incubated with 50 µg of DyLight 488-NHS dye at 21 °C and 600 rpm for 1 h using an Eppendorf thermomixer (ThermoFisher Scientific). Subsequently, samples were dialyzed extensively in PBS at 4 °C (buffer changed three times).

Size Exclusion Chromatography (SEC): The samples were analyzed on a Superose 6 increase 10/300 column (GE Healthcare). Analytical SEC measurements were executed on an Agilent 1260 bio-inert HPLC. Prior to injection, the samples were concentrated using centrifugal filters (Amicon Ultra 0.5 mL) to a final CP concentration of 100 µm. Samples were separated with a flow rate of 0.5 mL min⁻¹ in PBS.

Transmission Electron Microscopy (TEM): Protein samples were prepared with a final CP concentration of 10 µm. TEM grids (FCF-200 Cu, EMS) were gold-glared using a Cressington carbon coater and power unit. Protein samples (5 µL) were applied on the gold-glared grid and incubated for 1 min. The samples were gently removed using a filter paper. Finally, grids were negatively stained by incubation with 5 µL of 2% uranyl acetate solution in water for 15 s. After removal of the staining solutions, grids were allowed to dry for at least 15 min. The samples were analyzed on a FEI Tecnai 20 (type Sphera) equipped with a LaB6 filament and FEI B-CETA CCD camera at 200 kV.

Dynamic Light Scattering (DLS) Measurements: Samples (CP concentration 20 µm) were analyzed on a Malvern Zetasizer Nano ZSP. All measurements were performed at room temperature in triplicate and the average value reported.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE): Precast gels 4–20% (Bio-Rad) were used for sample analysis. The samples (5 µm final CP concentration) were prepared with 4x non-reducing loading buffer (Bio-Rad) containing 50 mm dithiothreitol (DTT) according to the manufacturer’s instructions. Gels were run in electrophoresis buffer (Bio-Rad) for 50 min at 150 V followed by staining with Coomassie-Brilliant Blue (Bio-Rad). Images were acquired with a GE Image Quant 350 (GE healthcare Europa GmbH).

Agarose Gel Electrophoresis (AGE): 1% agarose gels were prepared in TAE buffer containing SYBRGold. Samples (10 µm final CP concentration) were mixed with purple 6× (no SDS) gel loading dye (BioLabs) and run for 40 min 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).

Assessment of Loading Efficiency: The LNA loading efficiency of CCMV nanoparticles before and after cross-linking was assessed exploiting the Quant-IT RiboGreen RNA assay kit. Briefly, samples were dialyzed (3.5 kDa Spectra/Por dialysis tubing, Spectrum) and diluted in PBS to a final CP concentration of 0.2 µm (0.68 µg mL⁻¹ LNA positive controls) and incubated for 5 min with the RiboGreen reagent (1:1 v/v) protected from light. Fluorescence was measured (Ex/Emission: 475/520 nm) using a SPARK 10 m multimode microplate reader (Tecan). Statistical analysis (n = 3) was performed using a one-way ANOVA and Dunn’s test with GraphPad Prism 8.

 Extraction of Vitreous Humor from Porcine Eyes: The extraction of vitreous humor was performed as reported in the literature. Briefly, porcine eyes were obtained from the slaughterhouse (HKScan Finland Oyj) and processed within 24 h. After removal of extraocular tissues, eyes were quickly immersed in a 70% ethanol solution followed by overnight incubation in PBS at 4 °C. The dissection was performed by removing the anterior part of the eye, including the lens. The vitreous was collected and homogenized on an ice bath using a glass tissue homogenizer. This was followed by centrifugation at 1200 g for 1 h at 4 °C. The vitreous was filtered (0.45 and 0.22 µm Minisart hydrophilic regenerated cellulose syringe filters, Sartorius, Göttingen, Germany) and stored at −80 °C until use.

Stability Assay on CCMV Nanoparticles and Native Gel Electrophoresis: Samples for the stability study (20 µm initial CP concentration) were...
diluted in either PBS or vitreous humor to a final CP concentration of 5 μm and incubated at 37 °C. Prior sampling, the solutions were centrifuged twice at 12 100 g for 1 min using an Eppendorf MiniSpin (ThermoFisher Scientific) in order to precipitate aggregates. Aliquots (5 μL) were collected at different time points and analyzed through native gel electrophoresis. For this purpose, the samples, diluted in 2x non-denaturing loading buffer (62.5 mM Tris, 40% v/v glycerol, 0.01% bromophenol blue) were loaded on precast gels 4–20% (Bio-Rad) and run in electrophoresis buffer (192 mM glycine, 25 mM Tris, pH 7.5) for 1 h at 100 V. Gels were stained with SYBRGold and Coomassie-Brilliant Blue (Bio-Rad) and pictures acquired with a GE Image Quant 350 (GE healthcare Europa GmbH).

**Cell Viability Assay:** Mouse brain endothelial cells (bEnd.3 cells, purchased from ATCC) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin solution (P/S). The cells were seeded in a 96-well plate (5 × 104 cells per well) followed by incubation overnight at 37 °C in a 5% CO2 incubator. Samples (0.5–2 μm CP final concentration) were prepared in the presence of Lipofectamine 2000 (0.33% v/v) in FBS-free medium and incubated with cells for 12 h at 37 °C. The samples were replaced with fresh full DMEM and cells were incubated for an additional 24 h prior to analysis. Cell viability was assessed exploiting the alamarBlue assay kit according to the manufacturer’s instructions. Fluorescence was measured using a POLARStar Omega plate reader (BMG Labtech, Germany) at 544/590 nm. Statistical analysis (n = 4) was performed using a two-way ANOVA and Bonferroni test with GraphPad Prism 8.

**Cell Uptake Study:** bEnd.3 cells, cultured in DMEM (10% FBS and 1% P/S) were seeded in a 24-well plate (5 × 104 cells per well) followed by incubation overnight at 37 °C in a 5% CO2 incubator. The cells were incubated with labeled cross-linked LNA-loaded CCMV nanoparticles (0.25 μm final CP concentration) for 12 h at 37 °C. The cells were washed three times with PBS prior fixation in 2% paraformaldehyde solution (PFA) for 10 min at room temperature. After the fixation, the cells were blocked in 10% bovine serum albumin solution. Actin filaments were stained using Alexa Fluor 594 Phalloidin and cell nuclei using DAPI. Pictures were acquired using a Leica DMi8 inverted microscope (Leica Microsystems, Germany). Knockdown Efficacy Study: bEnd.3 cells, cultured in DMEM (10% FBS and 1% P/S), were seeded in 12-well plates (8 × 105 cells per well), followed by incubation overnight at 37 °C in a 5% CO2 incubator. The cells were incubated for 12 h with cross-linked LNA-loaded CCMV nanoparticles (0.25 μm final CP concentration, 103 nm LNA scramble, and 134 nm LNA anti-miR-23a/b) in the presence or absence of Lipofectamine in FBS-free medium. The samples were replaced with fresh full DMEM and the cells were incubated for an additional 24 h. Total RNA extraction was performed using the TRIzol reagent. The quality and quantity of isolated total RNA were determined. To convert microRNA to cDNA, reverse transcription was performed using the TRIzol reagent. The expression fold change of different gene (e.g., β-actin) was measured using a POLARStar Omega plate reader (BMG Labtech, Germany). The expression results were normalized to the 2^-ΔΔCt method. Statistical analysis (n = 3) was performed using a parametric unpaired t-test with GraphPad Prism 8.

**Supporting Information**

Supporting Information is available from the Wiley Online Library or from the author.

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**Conflict of Interest**

The authors declare no conflict of interest.

**Data Availability Statement**

Data available on request from the authors.

**Keywords**

antisense oligonucleotides, Cowpea chlorotic mottle virus, cross-linking, locked nucleic acid, miR-23, transfection, virus-like particles

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