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# Formulation and Physicochemical Characterization of NLC–miRNA Complexes

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## 1. Introduction

Although microRNA-based medicines have gained attention as a promising tool for the treatment of a variety of diseases, due to the poor biomembrane permeation, cellular uptake, and enzymatic instability of naked microRNA, the clinical success is mostly dependent on developing efficient and safe transfection vectors [1]. Therefore, we aimed to develop cationic nanostructured lipid carriers (cNLC) for targeting adipose tissue and delivering miR-27a.

## 2. Materials and methods

Stearylamine, Precirol® ATO 5, Miglyol® 812, Tween® 80, Pluronic® F68, and Milli-Q® water were obtained from Sigma-Aldrich (Germany), Herba Chemosan Apotheker-AG (Austria), Gattefossè Deutschland GmbH (Germany) Sigma-Aldrich (Germany), BASF (Germany), and Millipore S.A.S. (France), respectively. Double-stranded miRNA mimic mmu-miR-27a-3p (miR-27a) and serum-free low-glucose Dulbecco's modified Eagle medium (DMEM) were obtained from Dharmacon (GE Healthcare, Austria) and Gibco (UK). Nuclease-free water (VWR, Austria) was used to reduce the risk of nucleic acid degradation.

*Preparation of cNLCs by high-pressure homogenization process*

Stearylamine (0.15%; w/w), Precirol® ATO 5 (4.36%), and Miglyol® 812 (0.49%) were melted at 70°C. The surfactant solution, consisting of Tween® 80 (1%), Pluronic® F68(1%), and Milli-Q® water, was heated to the same temperature. Using the Ultra Turrax (IKA1-Werke GmbH & Co., Germany), the hot lipid phase was added to the aqueous phase, and the mixture was agitated for 60 s at 8000 rpm. The coarse emulsion was then homogenized three times at 70°C under a pressure of 800 bar (Panda 2K, NS1001L, GEA Niro Soavi, Germany).

*Preparation of CNE–miRNA complexes*

The cNLC–miR-27a complexes in the following mass ratios (expressed as SA/miR-27a) were prepared: 1:5, 1:2.5, 1:1, 2.5:1, 5:1, and 10:1. The miR-27a stock solution was diluted to a working solution of 1.3 μM with RNase-free water and combined in equal volumes with cNLCs, yielding a final miR-27a concentration of 650 nM. The complexation was performed at room temperature for 15-20 min.

*Stability studies of complexes depending on environmental changes*

To investigate the effect of the dilution medium on particle stability, the behaviour of the cNLC–miR-27a complex in zeta-water, RNase-free water, or serum-free low-glucose DMEM was monitored by dynamic light scattering (DLS) and electrophoretic light scattering (ELS).

*Particle size and zeta potential measurements*

The particle size and zeta potential (ZP) were determined using DLS and ELS technique with a Zetasizer Nano ZS (Malvern Instruments, UK).

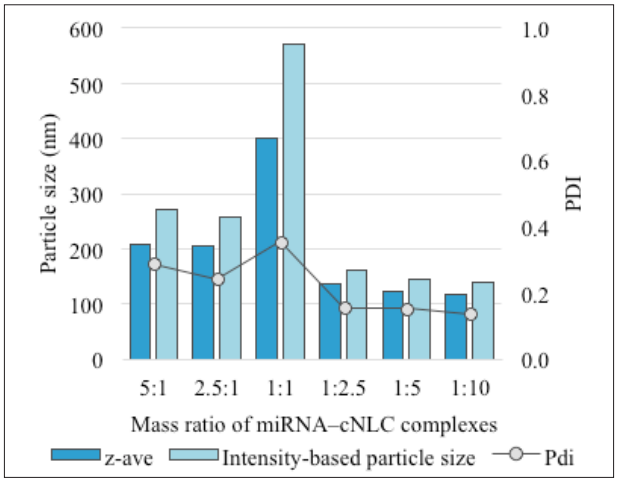


***Figure 1*** *Mass ratios of miRNA–cNLC complexes with different amounts of miR-27a*

The ZP measurements were repeated five times in disposable folded capillary cuvettes with a field strength of 20 V/cm.

*Gel retardation analysis of cNLC–miRNA complexes*

The gel electrophoresis was used to determine the eventual presence of unbound miRNA in complexes. The miR-27a and cNLC/miR-27a complexes were loaded into the appropriate wells and run on the 4% E-Gel™ agarose gel (Thermo Fisher Scientific Inc) for 15 minutes and observed using E-Gel Power Snap Electrophoresis System (Thermo Fisher Scientific Inc).

## 3. Results

To form a complex with miRNA, it is necessary to prepare cationic NLCs, and then incubate them with the miRNA. After homogenization, macroscopically homogenous milky-like formulations of NLCs with a relatively uniform size distribution were obtained (particle size of 102.27±0.45 nm and PDI of 0.17±0.01). A highly positive ZP value of +31.80±0.53 mV, indicate a well-charged droplet surface of cNLC, thus in principle promoting good physical stability.

*Physicochemical characterization of cNLC–miRNA complexes*

cNLC–miR-27a nanoparticles with various mass ratios are prepared with differing amounts of both components to examine their impact on particle size and ZP (***Figure 1***). For all tested mass ratios, the results show similar particle sizes ranging from 120 to 220 nm, whereby the intensity-based particle fraction slightly increased sizes of about 20-30 nm compared to the average value. These results show effective nanoparticle formation despite varying amounts of cNLCs and miR-27a. The biggest particle size was observed in the 1:1 ratio (400.8±24.7 nm) due to low ZP values (4.09±0.09 mV). Furthermore, the ZP increased from -20.5±0.6 mV to +28.5±0.5 mV. This invert from the negative to positive valuesuggests that miRNA started to locate at the particle interface.

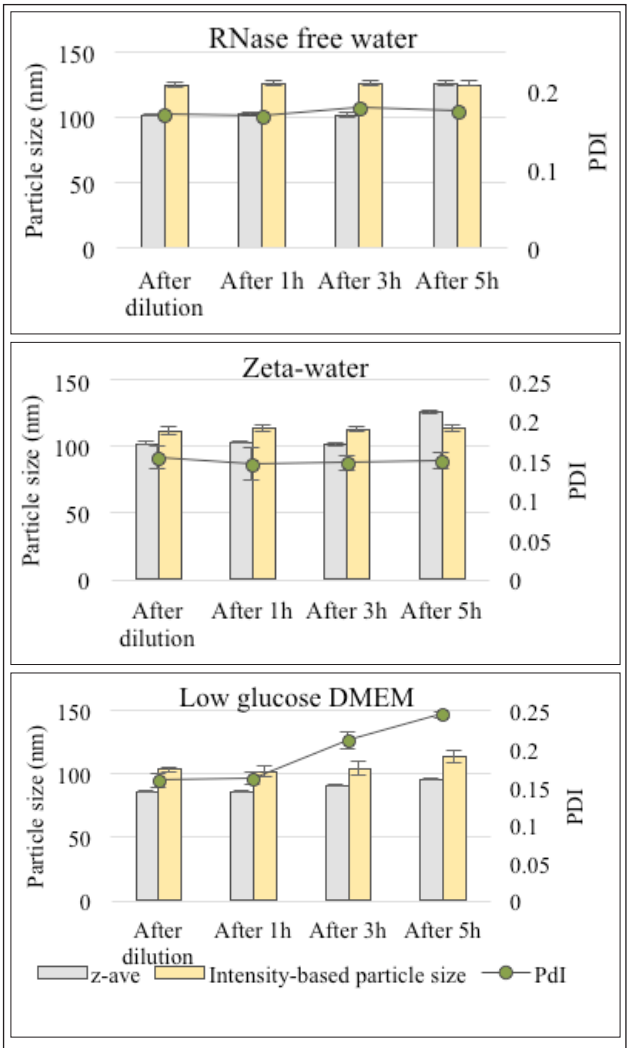


***Figure 2*** *Time–dependent DLS measurements of diluted cNLC complexes containing miRNA-27a. As dilution medium RNase-free water, zeta-water or serum-free low glucose DMEM are used*

*Stability depending on environmental changes*

The behaviour of cNLC–miRNA complexes in RNase-free water, zeta-water, or low glucose DMEM is investigated using DLS to determine the effect of the dilution medium on particle stability.

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For this purpose, standard cNLC–miR-27a complex (650 nM) is diluted immediately after preparation to obtain a miR-27a concentration of 100 nM. DLS measurements are summarized and displayed in ***Figure* 2**. Results have shown that in RNase free and zeta-water, the particle size and PDI values are relatively stable over time, whereas in low glucose DMEM the steady increase in z-ave, intensity-based particle size and PDI is observed. This phenomenon can be explained as low glucose DMEM containing amino acids, vitamins, and inorganic salts, which are expected to affect particle size and stability of the exposed cNLC–miRNA.

*Gel retardation analysis of cNLC–miRNA complexes*

Above a 1:5 mass ratio, gel electrophoresis revealed no unbounded miRNA, indicating that the cNLC–miR-27a complex formed successfully.

## 4. Conclusions

Physicochemical studies utilizing DLS, ELS, and gel electrophoresis showed that cNLC–miR-27a complexes are self-assembled. As a result, future investigations will be conducted in *in-vitro* cell culture model.

## 5. Acknowledgements

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## References

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