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Short communication

Functionalization of miRNA—cNLC complexes

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Introduction

MicroRNAs (miRNAs) are important regulators of gene expression in cells. However, their application in gene therapy is limited by obstacles such as poor cellular uptake and instability (Mendonça et al., 2023). To overcome these limitations, cationic nanostructured lipid carriers (cNLCs) as delivery systems for miRNAs are developed. cNLCs protect and stabilize miRNAs, and also enhance cellular uptake, which results in effective nucleic acid-based therapy. Another approach, found in literature, to enhance cellular uptake is coating particles with human serum albumin (HSA) (Liu et al., 2012). Therefore, the effect of functionalization of miRNA-cNLC complexes with HSA was investigated. The physicochemical properties of uncoated and HSA-coated complexes were compared in terms of particle size, size distribution, surface charge, topography, and cellular uptake in 3T3-L1 mouse embryonic fibroblasts and MCF-7 human breast cancer cells**.**

Materials and methods

Nanostructured lipid carriers (NLCs), composed of octadecylamine (OA), Precirol ATO 5, Miglyol 812, Tween 80, Poloxamer 188, and Milli-Q water were produced using a high-pressure homogenizer (70 °C, 800 bar, 4 cycles) as explained elsewhere (Tucak et al., 2020). The freshly produced cNLC formulation was used for complexation with miRNA mimic transfection control fluorescently labeled with Cy3 (FluoNTC) in a miRNA:OA mass ratio of 1:2.5 (w/w), followed by the gradual addition of human serum albumin (HSA) to produce HSA-coated miRNA-cNLC particles. The standard complexes, which contain 650 nM of miRNA, were further diluted with RNAse-free water (for DLS, ELS, and AFM analysis), or with phenol red-free low-glucose DMEM for in vitro experiments.

The particle size, size distribution, and zeta potential of coated and uncoated miRNA-cNLC particles were determined using a Zetasizer NS (Malvern Instruments, UK), while the morphology and topography of cargo-free NLCs, and coated and uncoated complexes were evaluated with a FlexAFM 5 atomic force microscope (Nanosurf, Switzerland).

The cellular uptake of coated and uncoated complexes was determined in two cell lines: 3T3-L1 mouse embryonic fibroblasts and MCF-7 human breast cancer cells, 4h and 24h after transfection. Cells were seeded with a seeding density of 7000 cells/well. Confluent cells were transfected using 200 nM of 1:2.5 (w/w) HAS-coated complex. After washing cells with PBS, and adding phenol-free low-glucose DMEM, cells were treated with 0.1% Triton X, and fluorescence intensity was measured using a CLARIOstar plate reader (BMG LABTECH, Germany). For confocal laser scanning microscopy, cells were seeded in glass-bottom dishes at a density of 7×10^4 cells. After transfection, cells were incubated for 4h and 24h and fixated using 4% formaldehyde. Cytoskeleton was counterstained using AlexaFlour 488, while nuclei were stained with Hoechst 33342 (Thermo Fisher Scientific, Austria). Images were obtained with Leica Stellaris 5 (Leica Microsystems, Germany).

Results and discussion

Prepared cargo-free cNLC formulation, miRNA-cNLC complex (1:2.5, w/w), and HSA-coated particles in

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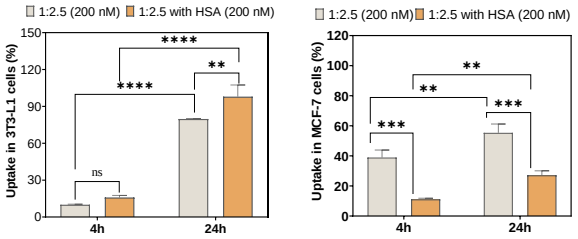
different mass ratios were characterized in terms of particle size, size distribution, and surface charge using DLS and ELS methods. The obtained results are presented in Table 1.

Table 1. Physicochemical characteristics of cNLC formulation, and complexes with and without HSA

Sample	Particle size (nm)	PdI	Zeta potential (mV)
cNLC	113.2 ± 1.7	0.213 ± 0.015	47.73 ± 3.52
miRNA-cNLC complex (1:2.5)	164.5 ± 11.9	0.220 ± 0.010	39.73 ± 0.66
miRNA:cNLC:HSA (1:2.5:10)	164.1 ± 39.2	0.230 ± 0.011	34.80 ± 0.17
miRNA:cNLC:HSA (1:2.5:100)	1631.1 ± 175.5	0.470 ± 0.075	2.94 ± 0.08
miRNA:cNLC:HSA (1:2.5:300)	1404.1 ± 158.7	0.510 ± 0.083	-6.60 ± 0.07
miRNA:cNLC:HSA (1:2.5:500)	870.2 ± 32.4	0.319 ± 0.014	-11.03 ± 0.21
miRNA:cNLC:HSA (1:2.5:700)	358.4 ± 1.5	0.208 ± 0.014	-13.80 ± 0.71
miRNA:cNLC:HSA (1:2.5:900)	315.8 ± 3.2	0.198 ± 0.013	-14.50 ± 0.50
miRNA:cNLC:HSA (1:2.5:1100)	152.3 ± 2.4	0.155 ± 0.008	-17.93 ± 1.18
miRNA:cNLC:HSA (1:2.5:1300)	142.4 ± 0.5	0.154 ± 0.005	-20.03 ± 0.12

As can be seen from Table 1, the particle size and PdI of cNLC formulation increased after the miRNA addition, while the zeta potential decreased. This can be explained due to the electrostatic interactions between cationic nanoparticles and negatively charged miRNA molecules, which caused miRNA molecules to be adsorbed at the particle's surface. However, after the gradual addition of HSA to the formed miRNA-cNLC complex (1:2.5, w/w), the particle size increased dramatically after the HAS addition in mass ratios from 1:2.5:100 to 1:2.5:300, after which it started to decrease to reach a size of ~140 nm. The changes in particle sizes are associated with the changes in zeta potential, as it decreased from around 34 mV to −20 mV, due to the addition of HSA, which is also negatively charged. In 3T3-L1 cells, 4h after transfection the uptake of complexes with and without HAS is relatively similar (around 10%), as shown in Fig.1. However, after 24 h, the cellular uptake of both types of particles is higher compared to cellular uptake after 4h, whereas a higher portion of HSA-coated particles was taken up in the cells (above 90% of particles). On the other hand, cellular uptake in MCF-7 followed a completely different uptake pattern. In brief, cellular uptake of particles without HSA was higher, after 4h and 24h, compared to HSA-coated particles. However, after 24 h, both types of cells were taken up in higher percentages, compared to those after 4h. These results are supported by confocal laser scanning microscopy.



a. b.

Fig. 1. Comparison of cellular uptake of FluoNTC—cNLC complexes (200 nM) at mass ratio 1:2.5 (w/w) and HSA-coated complexes at mass ratio 1:2.5:1300 (w/w) in a. 3T3-L1 cells and b. MCF-7 cells

Conclusion

After the successful production of cargo-free cNLC formulation, and complexation with miRNA molecules, it is necessary to optimize the concentration of HSA for coating the particles. Given that the physicochemical properties of coated and uncoated complexes are different in terms of particle size and surface charge, variations in cellular uptake can be expected. We have found that both transfection time and used cell line had a significant impact on cellular uptake. Therefore, these studies need to be performed in detail to explain which uptake mechanism is more dominant in these cell types.

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