Intracellular Delivery of Small Oligonucleotides with Lipid Nanocrystals (LNCs): *in vitro* studies

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Background

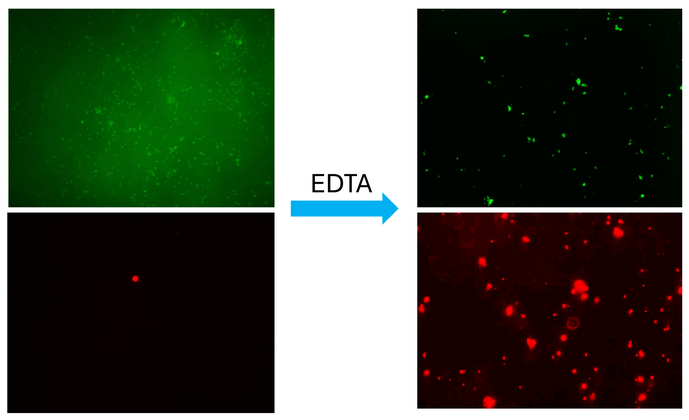
Since the approval of the first siRNA drug, Patisiran, in 2018, there has been ever-increasing attention to the opportunities for small oligonucleotide therapeutics in multiple therapeutic areas. However, major barriers to broader application have included limited options for delivery (largely systemic), toxicity, immunogenicity and challenges in reaching targets beyond the liver. LNCs are highly stable nanoparticles that self-assemble when phosphatidylserine (PS) liposomes and calcium are combined; their unique anhydrous multilayered structure permits incorporation of cargo molecules between the lipid bilayers. Outside of cells, normal extracellular levels of calcium maintain the crystalline LNC structure, while in tissues they are avidly taken up by professional phagocytes and injured cells. In the much lower calcium environment of the cytosol, LNCs lose their crystalline structure and release their cargo. LNCs have been used to successfully deliver amphotericin-B orally to patients with cryptococcal meningitis.

We sought to extend the application of LNCs beyond the delivery of small molecules in a series of in vitro studies in preparation for subsequent in vivo studies of LNCs for the oral delivery of small therapeutic oligonucleotides.

Methods and Results:

LNCs successfully encapsulate/protect RNAi (Fluorescence microscopy)

Recognizing the difficulties of encapsulating small oligos into lipid structures due to their charge and hydrophilic nature, we adopted formulation strategies that enabled both single-stranded ASOs and double stranded siRNA to be embedded in the LNC lipid layer, with excellent encapsulation efficiency (>90% by HPLC). Protection of the cargo within the crystalline LNC structure was further confirmed through fluorescence imaging with dual-labeled LNCs (lipid labeled with FITC; small oligo labeled with Cy5). Under fluorescent microscopy, only intact LNCs (green FITC) were visible. However, upon the addition of EDTA, both green FITC and red Cy5 were evident, confirming protection with intact LNCs, and release of cargo when calcium concentrations are lowered.

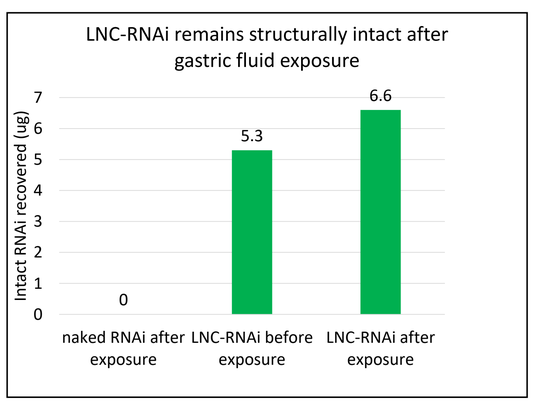


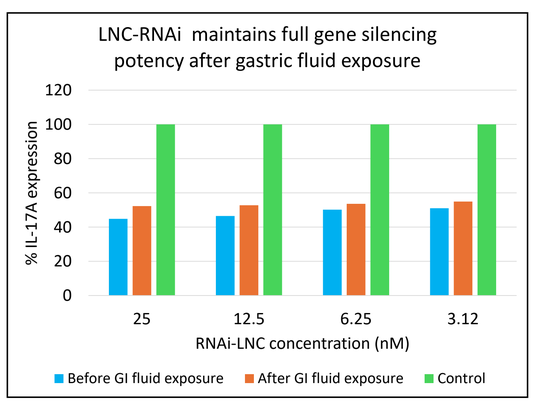
Before adding EDTA:
RNAi cargo (Cy5) is encapsulated in LNC and not visible

After adding EDTA:
Cargo release, both cargo (Cy5) and lipids (FITC) are visible

LNCs preserve the structural integrity and biological potency of oligonucleotides after simulated gastric fluid exposure (HPLC & gastrointestinal stability assay)

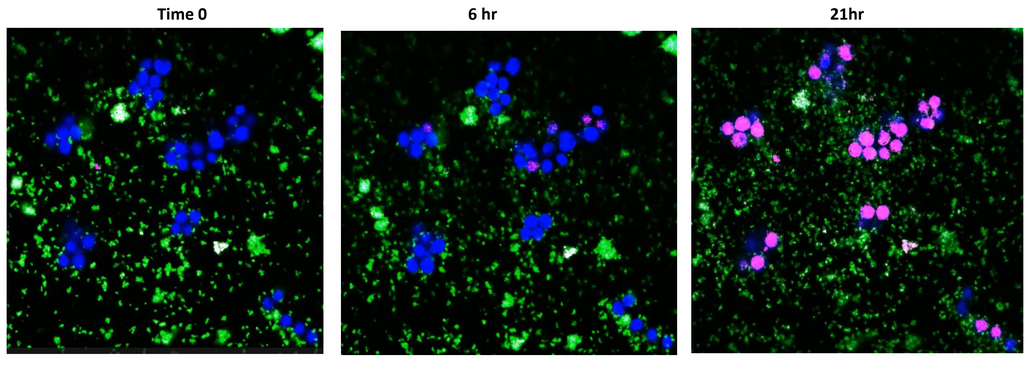
The LNC structure also provided robust protection for small oligonucleotides targeting specific cytokines in a simulated gastrointestinal (GI) stability test, where unprotected small oligonucleotides were rapidly degraded upon exposure to simulated gastric fluid (pH 1.2, with pepsin, exposed for 1 hour), while LNC-encapsulated small oligos remained intact, with no change in their cytokine knockdown capabilities after GI fluid exposure.





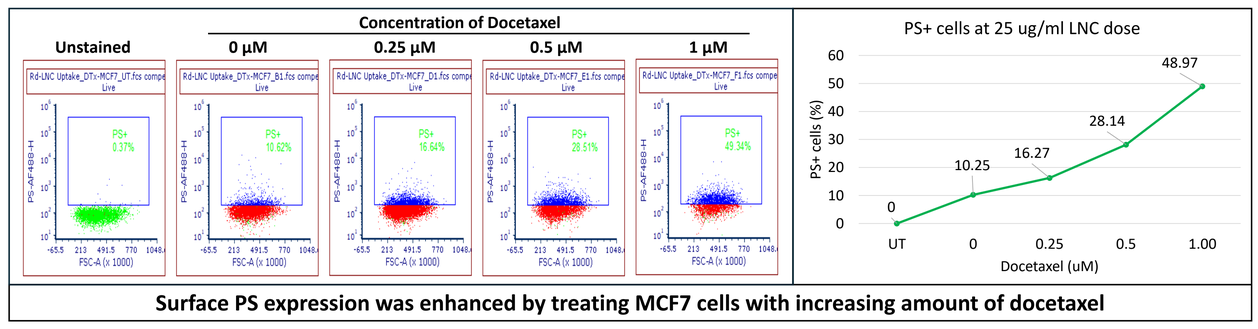
LNCs release oligonucleotides in response to the intracellular ultra-low calcium levels (Confocal microscopy)

We also conducted live cell imaging studies in HEK293 cells. Dual-labeled LNCs (256 ng/mL scrambled siRNA) were added, and cells imaged every 30 minutes for a period of 24 hours to examine the dynamics of LNC uptake in these cells. For higher-resolution mechanistic imaging, HEK293 cells were treated with LNCs and harvested at key time points followed by staining with LAMP1 with immediate imaging using a LSM confocal 780 microscope to examine endosomal uptake. Over a 24-hour time course, only green FITC-LNC was observed for the initial 5 hours, strongly suggesting that all siRNA was encapsulated, and no free nucleotides were present. From the 6th hour onward, more and more red Cy5 signals appeared inside the cells, indicating that LNCs had been taken up by the cells and that the oligo cargo was released only in the ultra-low calcium intracellular environments.

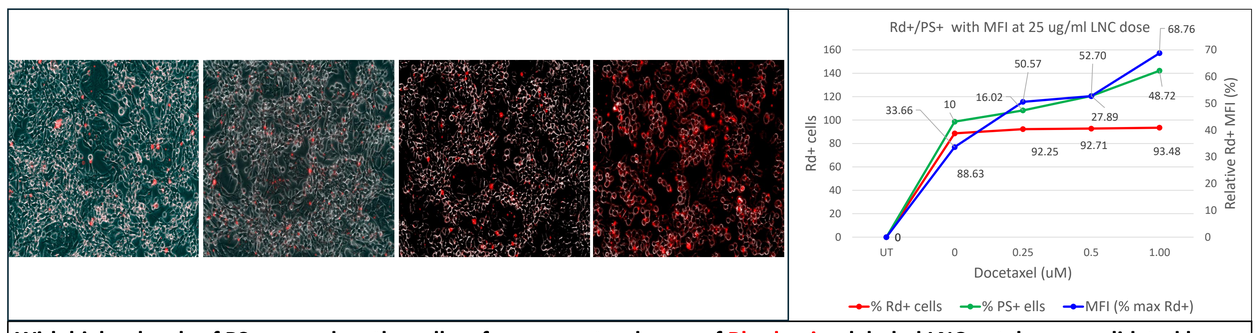


Phosphatidylserine on the outer cellular membrane drives LNC uptake

We assessed cellular targeting by measuring in vitro uptake of fluorescently (Rhodamine, Rd) labeled LNCs using both fluorescence microscopy (qualitative) and flow-cytometry (quantitative) in multiple somatic and immune cell lines, with both empty LNCs, and LNCs carrying different small oligo cargos, including a TNFα ASO, a scrambled siRNA, and 3 different 3'-UTR mRNA-targeted small oligos designed to knock down individual cytokines – specifically IL17A, TNFα, and IL-23p19. We noted more avid uptake in cells with higher surface phosphatidylserine (PS) expression; mechanistically this aligns well with the presumptive role of PS in both membrane fusion and efferocytotic phagocytosis.



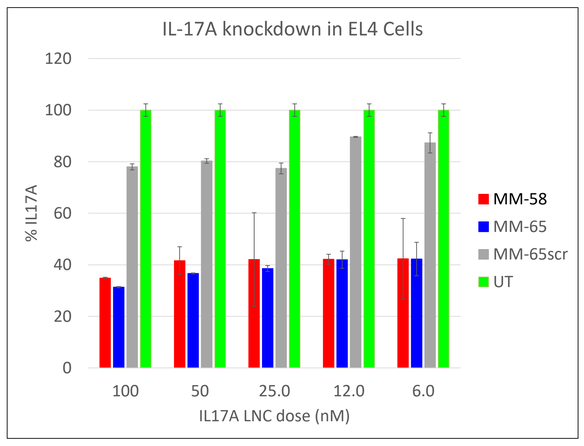
Surface PS expression was enhanced by treating MCF7 cells with increasing amount of docetaxel



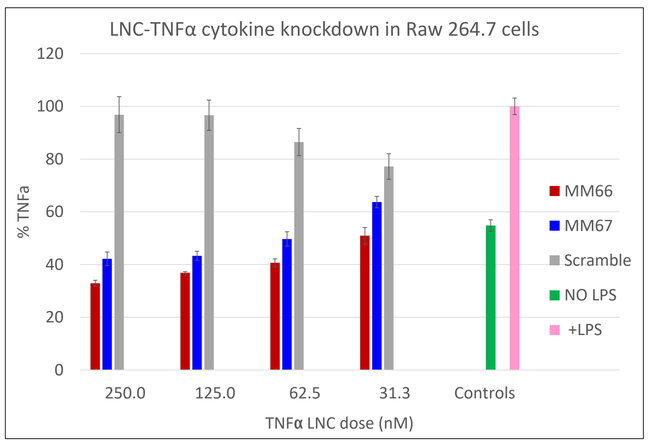
With higher levels of PS exposed on the cell surface, a greater degree of Rhodamine-labeled LNC uptake was validated by both fluorescent microscopy and flow cytometry

LNCs encapsulated therapeutic oligonucleotides showed robust knockdown potency in vitro

We then went on to perform *in vitro* cell culture studies with LNC formulations of different cytokine-targeted small oligos. Up to 70% of IL-17A cytokine knockdown was observed in EL4 cell lines, and similar potency of TNFα knockdown (around 70%) was observed in RAW264.7 murine macrophages using LPS stimulation to activate TNFα production, while scrambled small oligo formulation did not show much potency.



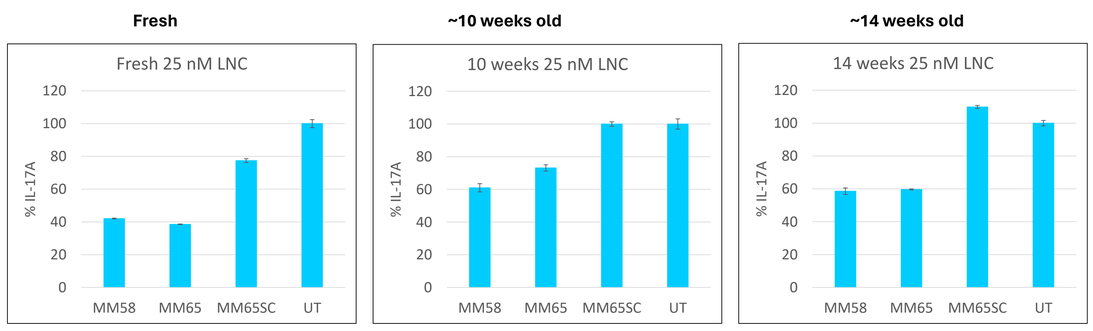
IL-17A cytokine knockdown in EL4 cells



TNFα cytokine knockdown in RAW 264.7 cells

LNCs showed superior stability at refrigerated (4°C) storage conditions for at least 14 weeks

When small oligo cargoes are encapsulated within the crystalline structure of LNCs, they are not only able to withstand degradation in challenging environments like gastric fluid but also exhibit remarkable storage stability over time. When stored under refrigeration at 4°C, these LNC formulations maintained their in vitro gene silencing potency for at least 14 weeks.



LNC-RNAi stable over 14 weeks at refrigerated storage conditions

Conclusions

LNCs appear to be a very promising new alternative for the oral delivery of small oligonucleotides:

1) LNCs successfully encapsulate/protect oligos, facilitate cellular uptake in high surface PS cells, release oligos efficiently in response to the intracellular ultra-low calcium levels.

2) LNCs preserve the structural integrity and biological potency of oligos in harsh environment.

3) Therapeutic oligos encapsulated within LNCs demonstrate robust knockdown efficiency across multiple cell lines, along with superior stability under refrigerated (4°C) storage conditions for at least 14 weeks.