Investigating the influence of manufacturing process of lipid-based formulations on intracellular delivery of siRNA

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Introduction

In recent years, nucleic acid (NA)-based medicines have emerged as a promising new biotherapeutic modality with the potential to approach targets previously considered 'undruggable' by small molecules. These could revolutionise the treatment of genetic disorders, infectious diseases and cancers. However, to be efficacious, they require a stable carrier (e.g. lipid-based formulation) capable of 1) protecting the NA cargo from enzymatic degradation; 2) preventing unwanted immunogenicity and off-target effects from naked NA and; 3) facilitating cellular trafficking and cytoplasmic delivery of intact NA to exert the desired therapeutic effect.

Liposomes were originally designed to improve the therapeutic index of small therapeutic molecules. Liposome composition has been enhanced over the years to improve stability, reduce drug leakage, and even to add stealth and targeting properties.

More recently, Lipid nanoparticles (LNPs) were specifically developed for delivering new modalities such as NAs. Like liposomes, LNPs protect and stabilise the cargo, and improve pharmacokinetics and cellular uptake. The key feature of LNPs is their suitability for intracellular delivery due to their ability to escape the endosomal compartment via the presence of the cationic/ionizable lipid.

The interplay between formulation and biological performance is more complex in NA-based therapies compared to conventional small molecule drug development and demands close collaboration between skill areas. Here, siRNA-loaded liposomes and LNPs were chosen as a case study to elucidate this formulation/efficacy relationship.

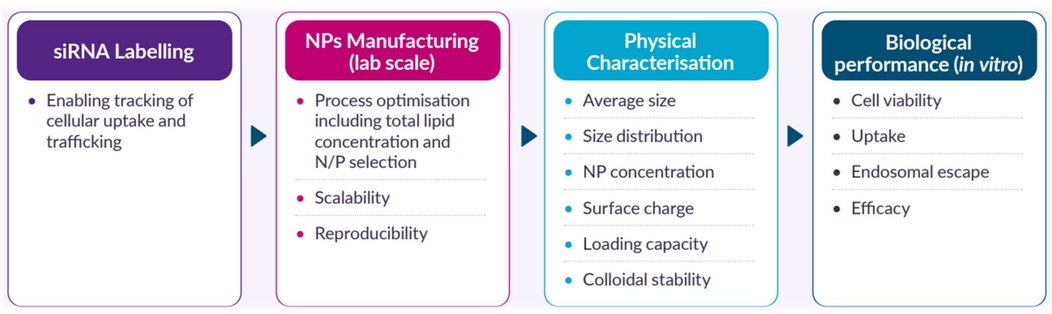

Fig. 1 Workflow from nanoparticle (NP) preparation to cellular analysis.

Experimental

Formulation manufacturing by conventional ethanol injection method

Lipoplexes and LNPs comprising the same lipids (i.e. DLin-MC3-DMA, (1,2-distearoyl-sn-glycero-3-phosphocholine) (DSPC), 1,2-dimyristoyl-rac-glycero-3-methoxypolyethylene glycol-2000 (DMG-PEG2000) and cholesterol with a ratio of 50:10:1.5:38.5) were manufactured with labelled siRNA (Cy3-siEHMT2) using a nitrogen/phosphate (N/P) ratio of 10. Manufacturing method differs only in the point of NA addition.

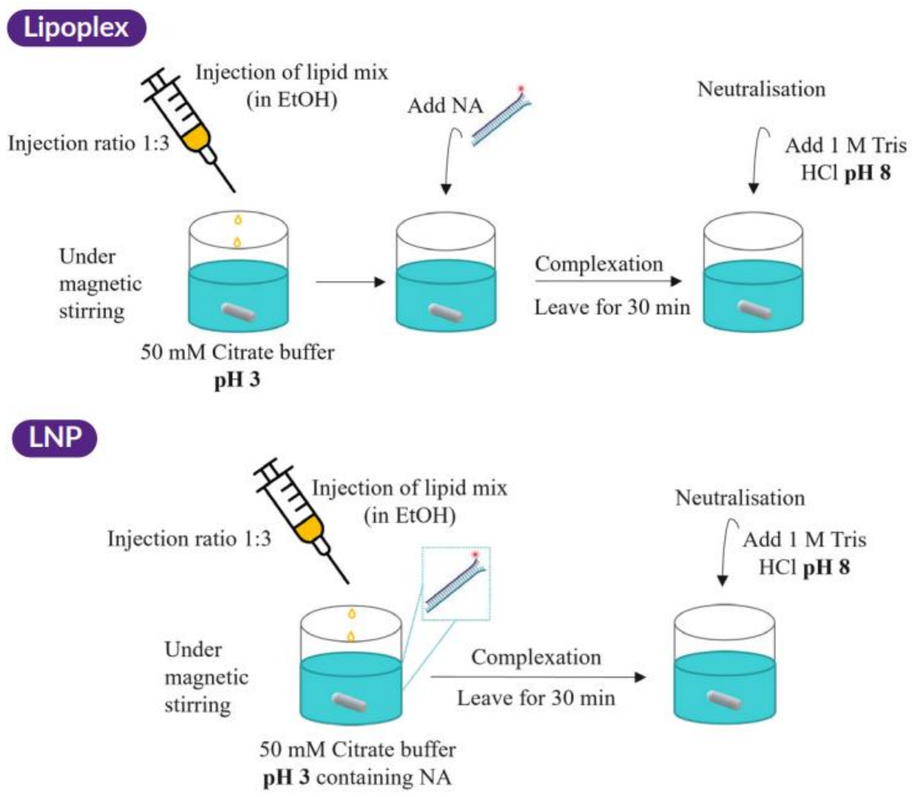

Fig 2. Shematic representation of the manufacturing process of the NA-loaded lipoplexes and LNPs

Physical characterisation performed by dynamic and electrophoretic light scattering (DLS and ELS) and single molecule localisation microscopy (SMLM), a super resolution microscopy technique improving resolution ten-fold compared to conventional widefield microscopy.

***In vitro* performance** evaluated using advanced microscopy techniques to demonstrate the kinetics of uptake, visualise trafficking and cytoplasmic delivery of siRNA by lipoplexes and LNPs.

Results and discussion

Physical characterisation of siRNA-loaded lipoplexes and LNPs

Similar physical properties between the lipoplexes and LNPs with z-av. of ~100 nm and PDI<0.2 indicating a narrow size distribution and neutral charge.

Excellent corroboration of the particle size determined by SMLM and DLS

Table 1. z-Average and PDI measured by DLS (n=3)

Property	Lipoplex	LNP
Z-average (nm)	113.5	105.7
PDI	0.16	0.15
Zeta potential (mV)	-2.3	-1.9
Cy3- siEHMT2 concentration (μM)	2.33	2

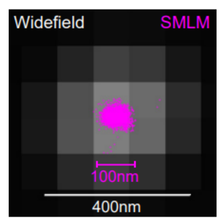

Fig 3. SMLM image of LNP

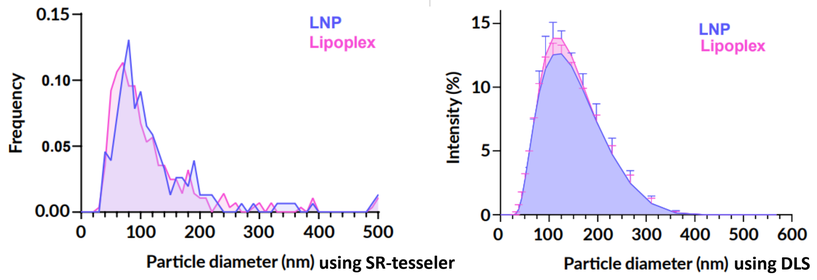

Fig 4. Particle size distribution

Localisation of siRNA within the two formulations

Encapsulation in the core of the LNP whereas in the lipoplexes cargo is both inside and outside of the lipid core.

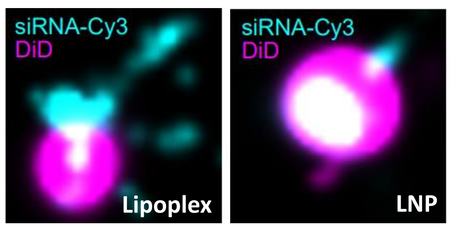

Fig 5. SMLM images

In vitro performance with HeLA cells at a siRNA dose of 100 mM

➢ Cellular uptake and internalization mechanism

Initial cellular uptake seen at 2 h and increased continuously to peak at 8 h for both formulations

No difference between the rate or level of uptake between the two formulations

LNP and Lipoplexes predominately internalised by Caveolae mediated endocytosis

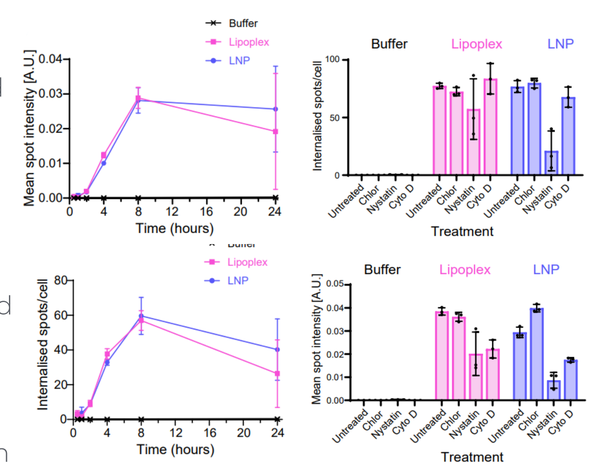

Fig 6. The number of siRNA-Cy3 spots inside the cell and the mean intensity of the siRNA-Cy3 spots over 24 h

➢ Endosomal escape

Rapid GAL9-eGFP recruitment initiating within 2 h and increasing to 24 h for both formulations

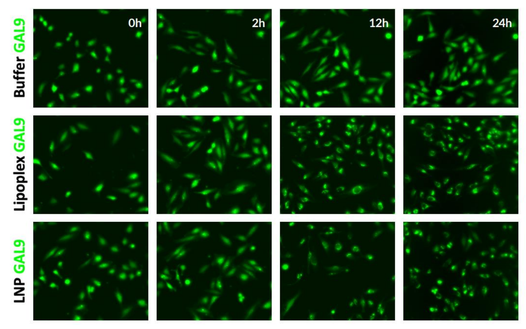

Fig 7. Representative images of endosomal rupture over time post treatment with LNPs and lipoplexes

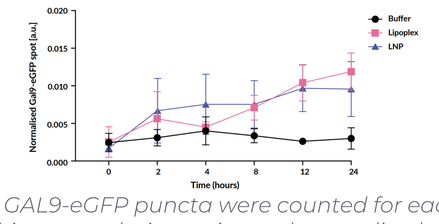

Fig 8. GAL9-eGFP puncta were counted for each condition at each timepoint and normalised to cell confluency.

➢ Transfection efficiency

Efficient knockdown of EHMT2 by both formulations

Efficiency comparable to commercial reagent with over 80% knockdown at 48 h

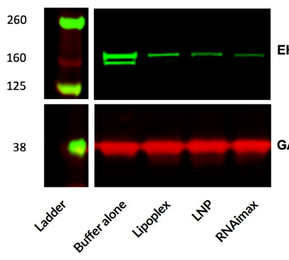

Fig 9. Western blot analysis of EHMT2 expression following transfection of HeLa cells with 100 nM of lipoplexes and LNPs for 48 h

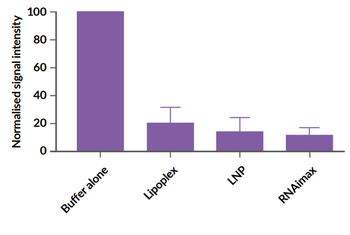

Fig 10. Normalised quantitative analysis of western blot data by densitometry analysis.

Conclusions

- Successful preparation of two lipid-based NP formulations using the same lipids and lipid composition, differing only in the point of NA addition, with similar colloidal characteristics
- Similar in vitro performance in HeLa cells of the siRNA-loaded lipoplexes and LNPs at a dose of 100 nM- this dose may exceed the optimal concentration for high transfection therefore obscuring potential difference in performance between the formulations
- A clear difference in the cargo interaction with lipids between the lipoplexes and LNPs was observed by SMLM. In an *in vivo* setting, NPs with surface exposed siRNA could exhibit altered biodistribution, immunogenicity or stability profiles compared with LNPS in which siRNA is encapsulated within the core.

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