MULTI-METHOD BENCHTOP ANALYSES TO GAIN NOVEL INSIGHT INTO STRUCTURE AND INTERNAL STABILITY OF LIPID NANOPARTICLES FOR MRNA DELIVERY

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Introduction: What are RNA and LNPs?

The spectacular success of the Covid-19 vaccines has brought global attention to the potential of mRNA as medicines. It has also helped emphasize, to the broader public, that there are other types of RNA with a huge therapeutic potential, such as short interfering RNA (siRNA). Although they are both polymers of ribonucleic acids, there are some notable differences. For example, mRNA is typically many hundred to several thousand monomers (nucleotides) long, whereas siRNA is typically in the range of 20-25 nucleotides. Furthermore, siRNA frequently contains numerous chemical (non-endogenous) modifications to improve its pharmacological properties. Nevertheless, a common delivery platform, lipid nanoparticles or LNPs, has proven its capacity to deliver both types of RNA, as demonstrated by the clinical introduction of LNP-encapsulated siRNA already in 2018 (Onpattro®), and lately by the Covid-19 mRNA vaccines.

LNPs (Figure 1) are generally composed of four main components: cholesterol, phospholipids, a PEG-conjugated lipid, and a synthetic cation-izable lipid. LNPs, generally with a diameter of 50–100 nm, are formed by controlled nanoprecipitation of the lipids around the RNA molecules. The existing models state that the ionizable lipid first surrounds the RNA by electrostatic interaction with the anionic phosphate groups. Subsequently, the cholesterol and phospholipids contribute as structural components, before the PEGylated lipid inserts into the LNP surface, with the PEG group facing outwards, providing a hydration layer, and making the LNPs less prone to early elimination by the immune system, increasing circulation time in the blood stream upon intravenous administration.

It is also very important to note that, in addition to their pharmacological function, both siRNA and mRNA are crucial structural components of the LNPs and together they form a non-covalent complex. With no RNA present, the LNPs are kept intact mainly by hydrophobic inter-lipid interactions, and their stability is severely reduced. Beyond this, however, there is considerable uncertainty about the detailed internal structure of LNPs.

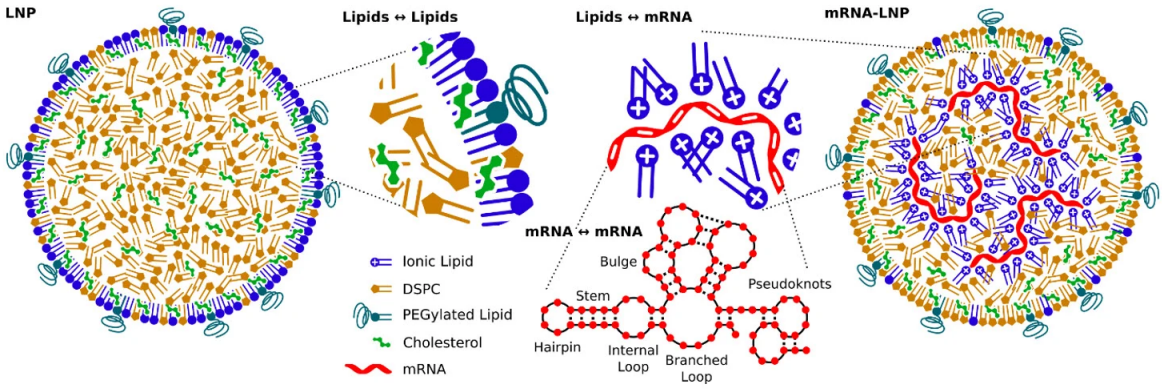


Figure 1. Schematic representation of a lipid nanoparticle empty (left) and encapsulating mRNA (right). mRNA can also form extensive secondary structures (lower middle). Inspired from Trollmann and Böckmann.,2022 [1].

Particle formation confirmed by size measurements

mRNA molecules are large (more than 600 kDa molecular weight) which are condensed when encapsulated into LNPs. The size, size distribution and polydispersity index (PdI) are indicators of the success of the particle formation. These values can be acquired using batch DLS which gives size and PdI information. Nevertheless, other more involved techniques, such as multi-detector-field flow fractionation [2] or microscopy (e.g., CryoTEM)[3], can provide orthogonal information regarding the size, size distribution, and real dispersity of mRNA-LNPs. mRNA molecules are considered as structure stabilizing compounds in mRNA-LNPs. Empty LNPs are not stable over time and always show higher PdI values(Figure 2). In addition to the final size and size distribution, the success of encapsulation of the mRNA by this delivery platform is influenced by the buffer composition used during and after the formulation process. This indicates that the understanding and control of internal structure and micro/nano environment inside the LNPs is very important to be able to understand and optimize the stability of such a delivery platform.

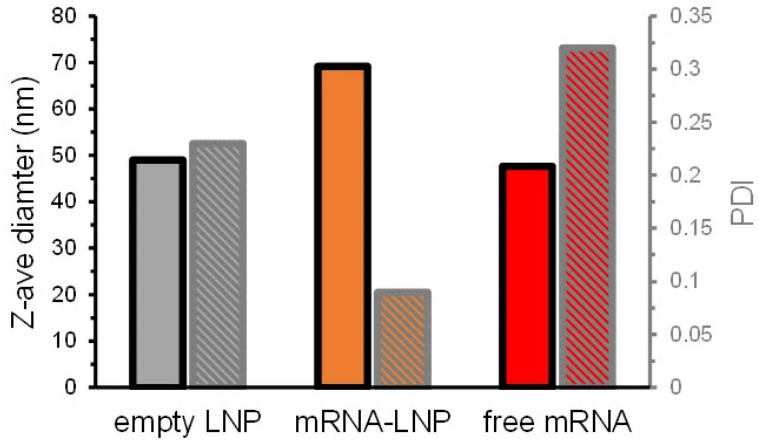


Figure 2. Size (Z-ave) and polydispersity index (PDI) of the empty LNP, free mRNA (Fluc mRNA), and mRNA-LNP. ZetaSizer DLS Ultra (Malvern Panalytical).

mRNA-LNP particles as supramolecular assemblies – the challenge of structural control

Beyond size and size distribution, structure and structural stability are the key attributes of a biotherapeutic drug determining its ability to consistently deliver and maintain the desired function throughout the manufacturing process, administration, and longer-term storage. This makes analysis of structure and stability into a critical task encompassing multiple physico-chemical and biochemical assays which together inform the selection of candidates and formulation conditions. While size and polydispersity measurements conducted over time or as a function of temperature can inform on stability of particles in mRNA-LNP samples, they need to be complemented by direct assessment of the intra-particle structure and structural stability of mRNA-LNP. Slight changes in formulation or storage conditions can affect the way the components interact and assemble into an mRNA-LNP complex [4,5].

What are CD spectroscopy and DSC, and why are they useful?

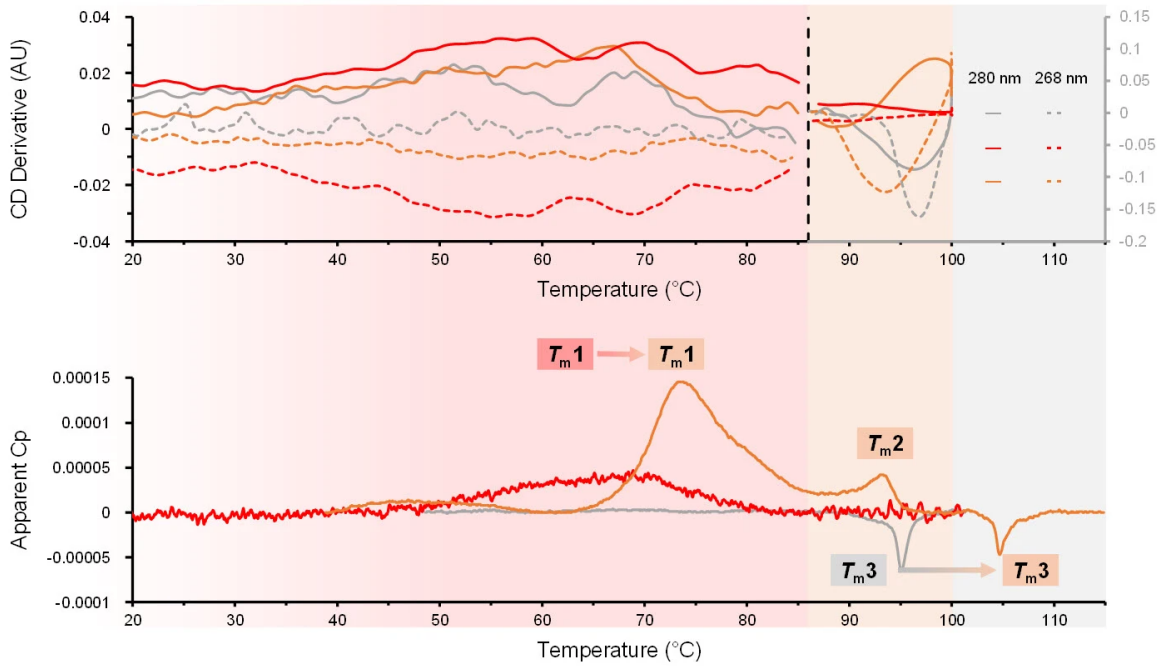
Circular Dichroism (CD) Spectroscopy and Differential Scanning Calorimetry (DSC) are two well-established tools that are broadly employed in the characterization of structure and structural stability during the development of biotherapeutics such as mAbs [4] and protein-based vaccines [5–7]. Changes in Tm values of thermal transitions detected by DSC and CD spectroscopy reflect intra- and inter-molecular interactions and alternations of the overall structure of biomolecules.

Technology	Measurement principle	Information content
CD spectroscopy	Measures the differential optical absorbance of right- and left-circularly-polarized light, as a function of wavelength and sample temperature [8]	State and changes in secondary and tertiary structure / higher-order structure of proteins and nucleic acids (non-destructive) and biomolecular stability through structural changes observed isothermally and during thermal ramps.
DSC	Measures the differential heat capacity of the sample during a thermal scan at a precisely controlled scan rate	Thermal stability and temperature-dependent changes in higher order structure of biomolecules and biomolecular assemblies, such as strand separation of nucleic acids, phase transitions of lipids and tertiary structure of protein domains, observed during thermal scans.

Higher order structure of mRNA-LNPs by CD spectroscopy and DSC

DSC and CD spectroscopy can be used to explore the structural complexity (morphology) of biomolecular assemblies such as mRNA-LNPs and to orthogonally inform on structural transitions related to mRNA. In addition, DSC can report on concomitant thermotropic transitions associated with the lipids forming LNPs. Figure 3 presents overlays of responses of free mRNA, LNP, and mRNA-LNP samples to a gradual increase of temperature monitored orthogonally with spectroscopic (CD) and calorimetric (DSC) readouts.

Firstly, several transitions could be repeatedly identified and linked to free mRNA or lipids-only based on the comparison of thermal fingerprints of these samples collected between 20ºC and 115ºC. The transitions showed marked differences in terms of cooperativity and sign. While the main transition detected for the free mRNA sample was endothermic and rather broad (non-cooperative), the transition observed for the lipid-only samples (LNP) was exothermic and rather narrow (cooperative). These characteristic transitions were also identified on the DSC traces of the mRNA-LNP samples with different levels of mRNA payload (data not shown).



T_m1	mRNA transition. The transition occurs at higher temperatures for mRNA in LNPs suggesting the encapsulated mRNA has higher stability towards heat-induced stress and its transition is affected by interaction with lipids
T_m2	Disruption of mRNA-lipid interaction detected by DSC and CD spectroscopy only in mRNA-LNPs samples
T_m3	Rearrangements in lipid partitioning and integration network. It is likely associated with the disintegration of LNP assemblies.

Figure 3. The lower figure presents an overlay of DSC thermograms of free mRNA, LNP, and mRNA-LNP samples marked with the transition ranges, Tms and trends. (MicroCal PEAQ-DSC automated; Malvern Panalytical). The upper figure presents numerical 1st derivatives of thermal ramp CD data for free mRNA, mRNA-LNPs, and empty LNPs. (Chirascan V100; Applied Photophysics).

Secondly, to validate the DSC findings, the same range of samples was subjected to CD thermal ramps and the resulting data was compared. Thermal transitions and ranges of thermal stability (Figure 3) identified by DSC agreed with the results of the CD thermal ramp measurements for mRNA, LNP and mRNA-LNP samples used in the present study.

Considering existing knowledge in the field, the data suggests that the structure of mRNA-LNP assembly is governed by a complex network of electrostatic, polar, and hydrophobic interactions between lipidic components and mRNA structural arrangement.

The extent of reversibility is another important aspect of the structural transitions observed in DSC and CD spectroscopy. Irreversibility of the thermal transitions of mRNA-LNPs observed in this study (Figure 4) corroborates the complexity of structure and interaction networks within the mRNA-lipid complexes and lipid phases resulting in kinetically controlled states and slow (if any) relaxation to the original structures. Additionally, the level of structural complexity is increased due to structural polymorphism of mRNA.

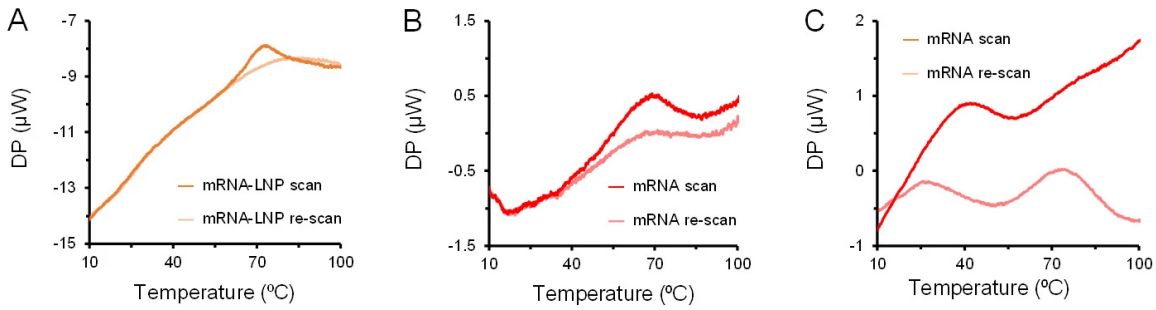


Figure 4. Overlays of raw DSC data as traces of differential power, DP, over temperature for a scan and a re-scan of (A) an mRNA-LNP sample with zero reversibility; and two different free mRNA lots with (B) partial reversibility; (C) structural polymorphism.

Where in the process of LNP drug development can DSC and CD spectroscopy be useful?

Compare morphology and structure of batches of drug substance and drug product

First principle label-free techniques, such as DSC and CD spectroscopy [7], are used for higher order structure and comparability analysis in accordance with FDA, EMEA, and ICH Guidance (Q6B, Q5E) and are established in development and QC of therapeutic mAbs [11,12] and nucleic acids including DS of Covid-19 vaccines.[13–15]. It is generally accepted that changes in DSC unfolding profiles and CD spectra may indicate changes in structural integrity and quality of drug substance and drug product. DSC is also used following the guidelines for physico-chemical analysis of liposome drug products where phase transition temperature of liposomal preparations is listed among important observables [16].

Due to the generic nature of DSC, calorimetric traces can serve as fingerprints of higher order structure of mRNA drug substance (Fig. 5a) and mRNA-LNP drug product assembly (Fig. 5b). Significant differences in the fingerprints detected with this easy-to-use technique can flag structural and/or chemical heterogeneity or signs of degradation leading to loss of function. These differences can be detected early on as long as other physico-chemical characteristics remain similar. The deviating samples can be then explored in detail with more involved dedicated techniques. DSC has been listed in the draft USP guidance among the methods for assessment of stability-indicating attributes of mRNA-LNP drug products under morphology [17].

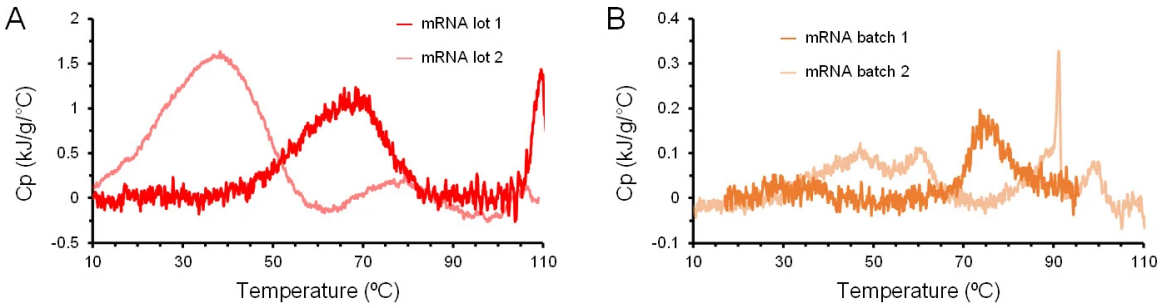


Figure 5. Overlay of DSC profiles for (a) two mRNA lots and (b) two batches of mRNA-LNPs. Batch 2 showed lower transfection efficiency.

CD spectroscopy is already well-established for the structural characterization of protein-based biotherapeutics and is routinely applied to nucleic acids, albeit more widely in academic research than in industry. However, the same approaches, as described below, can generally be applied to mRNA drug substances, DS, and already have been applied to DS of Covid-19 vaccines by major manufacturers [13–15].

For single-stranded oligonucleotides, CD spectroscopy yields signals due to stacking of base pairs in self-complementary sections that compose basic secondary structural elements (Fig. 1). Beyond these, CD spectroscopy can be used to identify different types of G-quadruplexes [18] that can form by stacking of guanosine residues in RNA, similarly as in DNA [19].

For double-stranded oligonucleotides, CD spectroscopy allows distinction between different helical conformations such as the right-handed A-form—which is characteristic for RNA—and B-form, and the left-handed Z-form [20–22].

With the aim of extracting more advanced information from CD spectra, there have been attempts to quantify the fraction of RNA base pairs [23], the fractions of G-quadruplex types [24], as well as a broader range of nucleic acid secondary structures [25], similar to secondary structure decomposition analysis for proteins.

As exemplified above (Fig. 3), insight into RNA stability can be obtained from thermal melting experiments [26], or by observing folding transitions, e.g., induced by addition of Mg^{2+} or urea [27].

Notably, absorbance is usually acquired simultaneously with CD. This allows, for example, for obtaining melting curves based on hyperchromicity [28], or accurate RNA quantification [29]. Generally, the extent of both base pairing and base composition affects the absorbance spectra of RNA. Furthermore, orthogonal fluorescence data might be obtained, for example, with assays based on fluorescent dyes or tagged RNA [30]. Modern CD systems offering corresponding accessories can provide rich datasets beyond CD data.

Informing analytical method development: mRNA-LNPs in presence of detergents

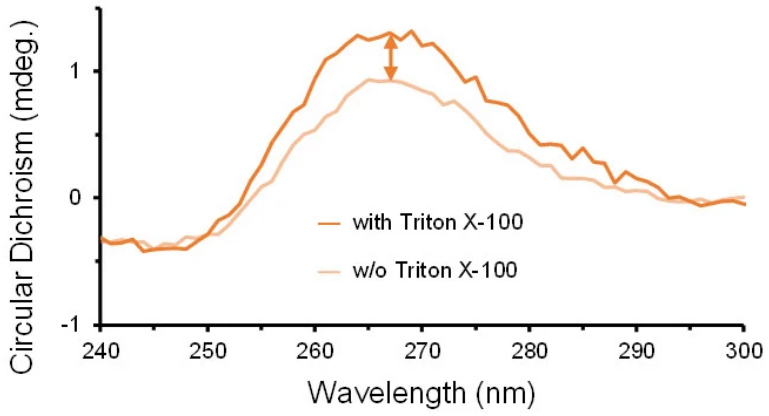


Figure 6. Overlay of CD data for mRNA-LNP sample in the absence and in the presence of Triton X-100 at otherwise identical conditions (Chirascan V100; Applied Photophysics).

Diversity of lipid formulations and mRNA payloads requires development of customized analytical methods. One of the well-known challenges in characterization of mRNA-LNPs is quantification of encapsulation level and payload. Methods based on the use of fluorescent probes are common and require use of detergents for mRNA extraction. The efficiency of extraction is likely to depend on the composition of the mRNA-LNP formulation and the extent of interactions within the particles. Therefore, there may be a need to customize the extraction conditions. CD spectroscopy can inform on the extraction efficiency and mRNA structural content. Figure 6 gives an example of a possible application. The presence of Triton X-100 increases the CD-signal of mRNA from LNP samples by ~30% as indicated by the ellipticity at 267 nm. This supposedly reflects a gain in structure due to reduced conformational constraints after solubilization. This, in turn, suggests that mRNA in LNPs, albeit stabilized, exists in a more moderately structured state. One might speculate that this is due to the dense lipidic environment imposing constraints on folding dynamics and, thus, preventing the more compact mRNA from probing the full energy landscape of potential conformations.

Perspectives on the use of CD spectroscopy and DSC

Beyond the already significant potential described above, there are several other applications where we foresee that DSC and CD spectroscopy could have significant impacts:

Double-stranded RNA (dsRNA) in mRNA secondary structures are formed through base complementarity (C–G and A–U) and subsequent strand annealing of two separate RNA strands, or internally by self-annealing of different sub-sequences within one RNA strand. For siRNA and other short RNAs, this is often a desirable or even prerequisite process, whereas for mRNA, these dsRNA structures are generally undesirable as they can be highly immunogenic and trigger severe adverse reactions. Importantly, the formation and maintenance of dsRNA structures are highly dynamic and dependent on, e.g., temperature, competing strand annealing, and the presence of other solutes, like conceivably also lipids. DSC and CD spectroscopy could inform the selection of optimal structures and conditions.

Shorter RNA, like antisense oligonucleotides or siRNA, are important therapeutics with some physicochemical properties that differ from mRNA, most notably their length and frequent chemical modifications. This translates into markedly different conditions for optimal encapsulation in LNPs, probably due to the structural function of RNA. Although some optimization principles have emerged, there is still a considerable need for a more detailed understanding.

Excipient composition and correlative analysis. The composition of the dispersant phase has a considerable impact on both LNP formation and stability. This includes factors like pH, presence of ions, and concentration of, e.g., cryoprotectants. Thus, DSC and CD spectroscopy could be used to both obtain fundamental understanding and to specifically optimize a drug product. It is notable that the escape of LNPs from the (acidic) cellular endosomes is considered one of the main hurdles to functional delivery of RNA. A correlative analysis of what affects structure and stability, e.g., by DSC and CD spectroscopy, with delivery and biological function (e.g., protein expression) in vitro in cells, could be a highly effective screening tool for the formulation of RNA.

Mapping of stability areas. Preliminary data such as in Figure 3 emphasize that LNP stability is highly dependent on the interactions between the individual compounds and could guide dedicated structural techniques, e.g., by identifying suitable temperature ranges for SAXS experiments. Considering conditions other than temperature and having a closer look at the lipid phase behavior in LNPs should prove even more insightful. This could be approached by generating pseudo-phase diagrams for mapping effects of, e.g., pH or the presence of detergent, on the different intermolecular interactions at play, and employing Isothermal Titration Calorimetry (ITC), which has already been successfully used to investigate lipid phase transitions [26,27], DNA-lipid interactions [28,29], and RNA-ligand interactions [30].

Conclusion

Complex structures of lipid-based vectors are formed through, and maintained by, interactions between their multiple components. Various stress conditions can impact physical and chemical integrity of the constituents of these biomolecular assemblies and affect the network of their interactions, and therefore the structure, stability, and ultimately the function of the entire delivery vector. Measurements of the impact of thermal stress on lipid-based vectors help to understand the behavior and structure of these biomolecular assemblies in solution and inform on rational approaches to their development, optimization of analytical assays, and design of stable liquid formulations. The current trend towards a systematic and detailed characterization of the structure of mRNA-LNPs is a prerequisite to robust product and process understanding and implementation of a Quality by Design approach. Biophysical techniques such as DSC and CD spectroscopy can be among the core tool set for mRNA-LNP development.

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