Experimental report

07/09/2022

Proposal: 9-13-1016 **Council:** 4/2021

Title: Understanding the influence of cholesterol on the ability of ionisable cationic lipid to interact with nucleic acid in lipid nanoparticles

Research area: Soft condensed matter

This proposal is a new proposal

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Samples: Cholesterol
MC3 Lipid (h- and d-forms)
poly A (nucleic acid)

Instrument	Requested days	Allocated days	From	To
FIGARO Langmuir trough	2	2	26/09/2021	28/09/2021

Abstract:

Lipid nanoparticles (LNPs) are increasingly being used to deliver nucleic acids and have reached world-wide prominence as the delivery vehicle for the COVID-19 vaccines produced by Moderna and Pfizer/Biontech. Yet few, if any, studies have examined the driving force for LNP self-assembly and, in particular, the structures formed as a result of the interaction of the nucleic acid cargo with the lipids - generally MC3 and cholesterol - which are believed to comprise the interior of the LNPs. Using the convenient platform of a Langmuir trough, preliminary surface pressure-area isotherms have shown strong effects of model cationic ionisable lipid monolayers without/with cholesterol and both without/with nucleic acid in the subphase. The present study will exploit neutron reflectivity measurements on FIGARO to understand the effect of the helper lipid cholesterol on the ability of the cationic ionisable MC3 lipid to interact with nucleic acid polyA. Feeding into the EU InnovaXN_25_MSCA_COFUND programme between AstraZeneca, University of Manchester and the ILL, the overarching goal of this project is to aid the development of improved lipid nanoparticles for future delivery systems.

<u>Final Experiment Report</u>: Understanding the influence of cholesterol on the ability of ionisable cationic lipid to interact with nucleic acid in lipid nanoparticles (#9-13-1016)

Introduction

Lipid nanoparticles (LNPs) have been increasingly studied as a means to effectively deliver nucleic acids to cell membranes in drug or gene delivery applications. Recently LNPs have reached world-wide prominence as the delivery vehicle for the COVID-19 vaccines produced by Moderna and Pfizer/Biontech [1]. LNPs contain as their cargo a nucleic acid, most commonly RNA, and are prepared from 4 lipids, namely an ionisable cationic lipid (typically (6Z,9Z,28Z,31Z)-heptatriacont-6,9,28,31-tetraene-19-yl 4-(dimethyl-amino)butanoate, **DLin-MC3-DMA or MC3**), a phospholipid (1,2-distearoyl-sn-glycero-3-phosphocholine, **DSPC**), a neutral helper lipid (**cholesterol; chol**) and a (negatively charged) pegylated lipid (most generally **DMG-PEG-2000**). The most widely studied LNP consists of MC3:DSPC:chol:DMG-PEG-2000 in the molar ratio 50:10:38.5:1.5 [2].

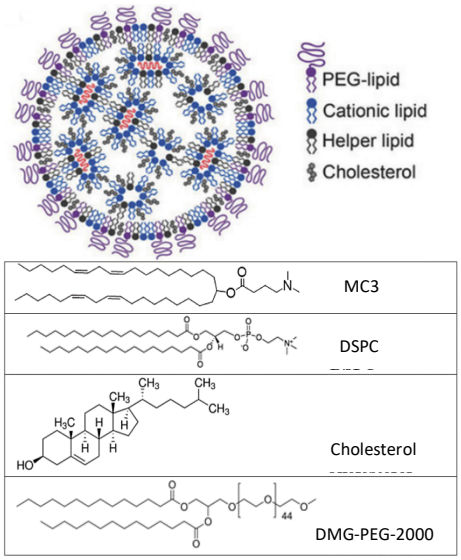

Fig. 1 *Schematic representation of a lipid nanoparticle alongside key chemical component structures.*

LNPs are prepared by dissolving (i.e. molecularly dispersing) the lipids in ethanol, and then mixing using microfluidics with an aqueous solution of nucleic acid at pH 3.0 such that the final volume of ethanol is less than 25%. The LNPs thus formed are then dialysed against PBS buffer at pH 7.4. The lipids are considered to self-assemble to form the LNPs upon contact with the aqueous solution containing RNA. Significantly, within the LNPs there is a phase separation of lipids such that nucleic acid is contained in the interior along with the ionisable cationic lipid (MC3) and some cholesterol, while the DMG-PEG-2000, DSPC and remaining cholesterol are expressed in the exterior [3]. Arteta et al [3] estimated that the molar ratio of ionisable cationic lipid:cholesterol present in the LNP core was ~76:24 (lower than the ~56:44 ratio commonly employed for LNP preparation) suggesting that some of the cholesterol helper lipid is involved in the interaction between the ionisable cationic lipid and nucleic acid payload.

Experiment Details

Due to the misalignment problems experienced in beam time experiment #8-02-944, which involved a small Langmuir trough in the centre of the sample stage, the proposal of this experiment (#9-13-1016) focussed only on collecting structural datasets. Specifically, in 4 contrasts (d-lipid/polyA and h-lipid/polyA each in ACMW and D_2O) over the full Q-range at a starting surface pressure of 25 mN/m. However, due to efforts by the PhD student, the injection trough was improved such that a new Teflon trough was cut and positioned nearer to the beam-defining slits, with 2 additional holes for sample injection (6 total) for more uniform mixing of the nucleic acid. This gave us the confidence in the experimental setup to collect low-Q data sufficient for a kinetics analysis which was highly desirable due to the greater insight into the self-assembly mechanism. Studying these kinetics meant that each contrast took ~1 hr longer, which made it impossible to study all 4 systems in 2 days of allotted beamtime. Consequently, we decided to measure 4 contrasts (2x lipid, 2x solvent) of MC3 and MC3:chol in PBS, and MC3 in citrate along with pure lipid reference measurements for MC3 in PBS (4 contrasts). Despite successful measurements, the MC3:chol in citrate and key reference measurements are missing. Direct beams and pure solvent calibrations were run for a total of 8 hours.

Data Analysis – Structure

The structural data following the interaction of 0.08 mg/ml of the nucleic acid, the adenosine monophosphate homopolymer, polyadenylic acid (polyA) administered into the sub-phase via syringe pump injection (1 ml/min) are presented in Figure 2. Measurements were recorded on a Langmuir trough at a starting surface pressure of 25 mN/m close to physiologically relevant values both in the absence and presence of 25 mol% cholesterol on a sub-phase of 0.01M phosphate-buffered saline (PBS, pH 7.4) and 100 mol% MC3 on 0.05 M citrate buffer (pH 3.0) (to mimic dialysis and self-assembly stages during fabrication respectively). This concentration was selected as it has been used successfully to study the interaction of cationic lipids used to prepare gene delivery vehicles with nucleic acids [4].

Fully deuterated (d45-) cholesterol was sourced from ANSTO and d62-MC3 prepared using established protocols by AstraZeneca (AZ) to enable significant sample deuteration, exploiting the EU InnovaXN_25_MSCA_COFUND programme between AZ and the University of Manchester. This allowed us to study four contrasts (d- and h- lipid on D_2O and ACMW), as shown in panels 2(a-c). These obtained data have been modelled with a chains layer of 11.8 Å, a head group layer of 6.0 Å with 52.9% solvent, and a nucleic acid layer of 20.0 Å (Dabkowska et al [5]) with 92.2% solvent. Table 1 shows the fitting parameters and a schematic in Figure 2 highlights the observed differences between the systems. Despite the reasonable fit, it is not possible to accurately quantify the changes to the monolayer in each contrast without the reference measurements. In particular, the d-lipid in D_2O model shows consistent deviation from the data in all systems above Q = 0.10 $Å^{-1}$. Figure 1(d) shows the surface excess kinetics of polyA after injection (t = 0), where at low pH the nucleic acid is observed to immediately interact with the MC3 monolayer, while at a higher pH it takes ~30 min to reach a similar level. When cholesterol is present, there is a reduced

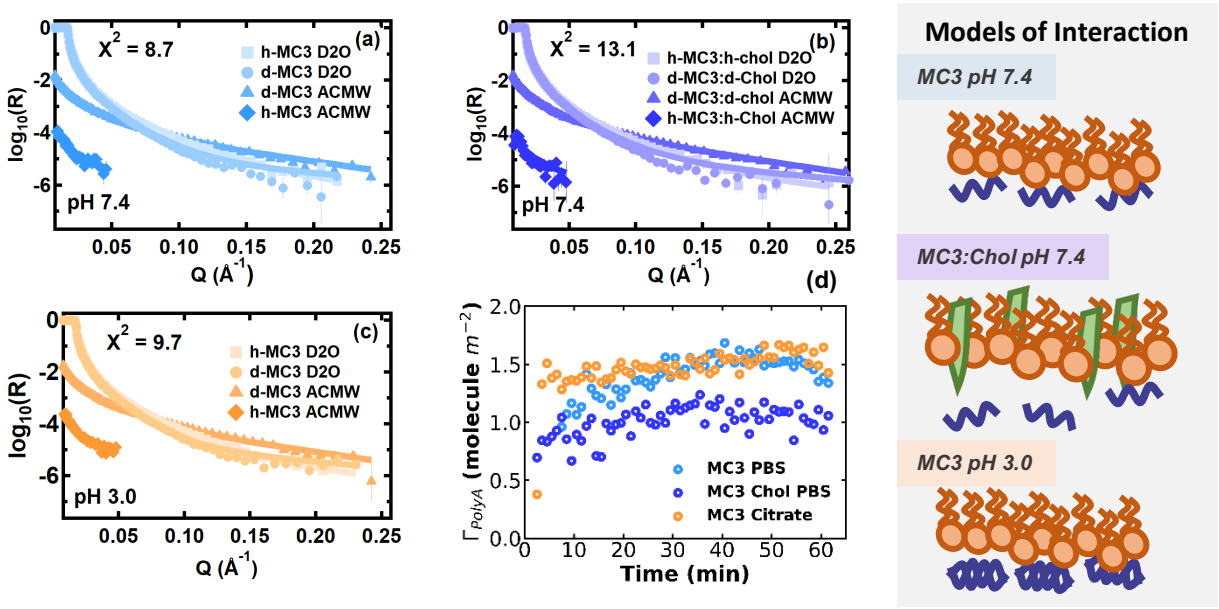

Fig. 2 *Structural neutron reflectivity against Q for (a) MC3; (b) MC3:chol (75:25) monolayers on 0.01 M phosphate buffered saline (pH 7.4); (c) MC3 on citrate (pH 3.0) buffer. Surface excess of polyA from point of injection (d). Schematics depicting models of the interactions are shown on the right. Monolayers were spread to P = 25 mN/m on 45 ml of subphase prior to the injection of 5 ml of subphase containing polyA [0.56 mg/ml] at 1 ml/min for a final trough-polyA concentration of 0.08 mg/ml. All measurements were performed at room temperature.*

Table 1 *Associated model parameters where chain-layer thickness and backing SLD in D_2O were fitted, head-layer thickness was fixed, and the nucleic acid layer was fixed at 20 Å with associated solvent fitted. The model features an average chain length of 11.842 Å across the three systems, with a head group thickness of 6.0 Å. Parameters give a reasonable fit in all contrasts except when deuterated cholesterol is present in D_2O from Q ~ 0.10 $Å^{-1}$.*

Contrast	pH	Backing SLD	Roughness	Chain Thickness / Å	Chain SLD	Head Thickness / Å	Head SLD	%Head Solvent
hMC3 D_2O	7.4	6.150	3.5	11.388	-0.073	6.0	0.726	52.1
dMC3 D_2O		6.084			6.193			
hMC3 ACMW		0			-0.073			
dMC3 ACMW		0			6.193			
hMC3:hChol D_2O	7.4	5.978	3.5	11.724	-0.031	6.0	0.748	58.7
dMC3:dChol D_2O		6.121			6.444			
hMC3:hChol ACMW		0			-0.031			
dMC3:dChol ACMW		0			6.444			
hMC3 D_2O	3.0	6.080	3.5	12.413	0.726	6.0	0.726	47.8
dMC3 D_2O		6.069			6.193			
hMC3 ACMW		0			0.726			
dMC3 ACMW		0			6.193			

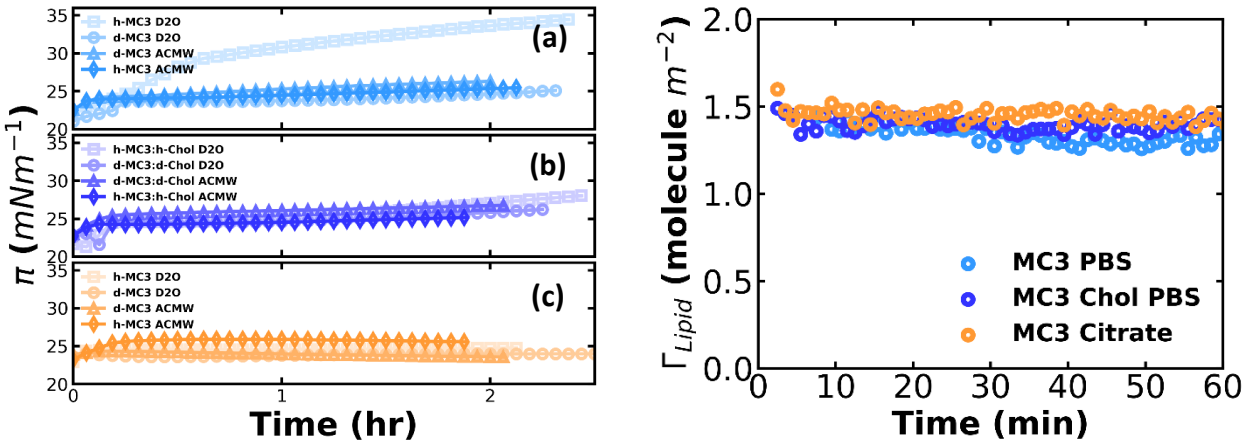

Fig. 3 *(Left) Surface pressure, π, injection kinetics for MC3 in PBS pH 7.4 (a), MC3:chol (75:25 mol%) in PBS pH 7.4 (b) and MC3 in citrate pH 3.0 with polyA in 50 ml of D_2O or ACMW (8.1% v/v D_2O). Monolayers were spread to P = 25 mN/m on 45 ml of subphase prior to the injection of 5 ml of subphase containing polyA [0.56 mg/ml] at 1 ml/min for a final trough-polyA concentration of 0.056 mg/ml. All measurements were performed at room temperature. (Right) Lipid surface excess from point of injection.*

polyA interaction with the monolayer. The known pH dependent behaviour of polyA, which transitions from a single to double stranded helical structure as pH decreases from neutral to acidic [6], is thought to contribute to the differences in these interactions. Since the experiment, we have established that this behaviour contributed to the aggregation of nucleic acid during sample preparation. This leads us to believe that the size of the polyA interacting with the monolayer was greater than expected which raises the question of the effect of nucleic acid aggregation on LNP self-assembly. Further study of this phenomenon via neutron reflectometry is essential to understanding and publishing the collected data.

Data Analysis – Surface Pressure & Excess

In addition to the neutron reflectivity data, we simultaneously recorded surface pressure. The data in Figure 3 (left) show the evolution of the system following injection of the polyA at t=0, the subsequent kinetics, and equilibrated structure. The first hour was allotted to kinetics which appear to be constant over time in all contrasts except h-MC3 in PBS D_2O buffer, Figure 3a, which displays an unexplained anomalous increase in surface pressure. The modest increase in the surface pressure suggests that the polyA did not significantly penetrate the monolayer, which is supported by the structural fits presented in Figure 2(a-c) where a layer of nucleic acid was modelled beneath the head group, giving reasonable χ^2 values. The lipid surface excess (Figure 3, right) was also observed to be constant, indicating that no lipids entered the subphase upon interaction with the polyA, further supporting the stable surface pressure.

As in experiment #8-02-944, the sample environment was saturated by placing dishes of D2O/ACMW around the trough to reduce the effects of evaporation. This precaution, alongside working in a sealed environment, was essential in preventing against the effects of oxidation on MC3 which has two double bonds in the tails, as shown in Figure 1. Further work on elucidating these interactions by deliberately flowing oxygen over the monolayers in situ is necessary for publication of the complete system.

Outlook

We were able to record 4 contrasts (2x lipid, 2x solvent) in three out of four systems, allowing us to study the interaction of the nucleic acid with the key components present in the interior core of the LNP, and 4 lipid reference contrasts (2x lipid, 2x solvent) at pH 7.4. Additional neutron reflectivity data are required to collect the final system, 4 contrasts of MC3:chol at pH 3.0 with nucleic acid, as well as 16 pure lipid reference measurements (2 systems x2 pH x 4 contrasts), to fully elucidate the mechanism(s) of interaction and provide us with enough confidence to publish the collected data. Follow-up measurements focused on the effects of nucleic acid aggregation and MC3 oxidation are also essential to publishing a complete characterization.

References

[1] Buschmann et al (2021) Vaccines 9 65; [2] Semple et al (2010) Nat Biotech 28 172; [3] Arteta et al (2018) PNAS 115 E3351: [4] Dabkowska et al (2011) J Roy Soc Inter 9 548; [5] Dabkowska et al (2012) Biomacromolecules 13(8):2391-401; [6] Ke et al (2009) Biophys J 96 2918.