EUROPEAN SYNCHROTRON RADIATION FACILITY

INSTALLATION EUROPEENNE DE RAYONNEMENT SYNCHROTRON

ESRF

Experiment Report Form

The double page inside this form is to be filled in by all users or groups of users who have had access to beam time for measurements at the ESRF.

Once completed, the report should be submitted electronically to the User Office via the User Portal:
https://wwws.esrf.fr/misapps/SMISWebClient/protected/welcome.do

Deadlines for submission of Experimental Reports

Experimental reports must be submitted within the period of 3 months after the end of the experiment.

Experiment Report supporting a new proposal ("relevant report")

If you are submitting a proposal for a new project, or to continue a project for which you have previously been allocated beam time, you must submit a report on each of your previous measurement(s):

- even on those carried out close to the proposal submission deadline (it can be a *"preliminary report"),*
- even for experiments whose scientific area is different form the scientific area of the new proposal,
- carried out on CRG beamlines.

You must then register the report(s) as "relevant report(s)" in the new application form for beam time.

Deadlines for submitting a report supporting a new proposal

- 1st March Proposal Round - **5th March**
- 10th September Proposal Round - **13th September**

The Review Committees reserve the right to reject new proposals from groups who have not reported on the use of beam time allocated previously.

Reports on experiments relating to long term projects

Proposers awarded beam time for a long term project are required to submit an interim report at the end of each year, irrespective of the number of shifts of beam time they have used.

Published papers

All users must give proper credit to ESRF staff members and proper mention to ESRF facilities which were essential for the results described in any ensuing publication. Further, they are obliged to send to the Joint ESRF/ ILL library the complete reference and the abstract of all papers appearing in print, and resulting from the use of the ESRF.

Should you wish to make more general comments on the experiment, please note them on the User Evaluation Form, and send both the Report and the Evaluation Form to the User Office.

Instructions for preparing your Report

- fill in a separate form for each project or series of measurements.
- type your report in English.
- include the experiment number to which the report refers.
- make sure that the text, tables and figures fit into the space available.
- if your work is published or is in press, you may prefer to paste in the abstract, and add full reference details. If the abstract is in a language other than English, please include an English translation.

ESRF	**Experiment title:** Colloidal Transitions During pH-Triggered Deprotection of Fatty Acids in Nanocarriers for siRNA Delivery	**Experiment number**: A26-2-955
Beamline: BM26	**Date of experiment**: from: 17.02.2023 to: 20.02.2023	**Date of report**: 05.05.2023
Shifts: 9	**Local contact(s)**: Martin Rosenthal	*Received at ESRF:*

Names and affiliations of applicants (* indicates experimentalists):

Prof. Bruno De Geest

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Report:

Introduction

To improve the efficiency of siRNA delivery, we have synthesized a small library of lipids with dual pH-responsive behavior that can be incorporated into lipid nanoparticles (LNPs) (Fig. 1). When triggered by low pH conditions, these lipid molecules release fatty acids inside the LNPs, causing nanostructural rearrangements and providing new pH-responsive capacities. We were interested in following colloidal and nanostructural changes upon changing the lipid composition in these LNPs either by changing the formulation (mixing different ratios of lipids) or as a result of pH-triggered degradation.

Methods

Because fatty acids are created as a result of the pH-triggered degradation, special attention was needed to keep the pH of the reaction constant. The experimental design initially envisioned relied on a flow-through setup where the LNPs would reside in a reaction vessel (eppendorf tube) and would be contunuously pumped into the X-ray beam through a flow-through capillary by means of a peristaltic pump. The would allow us to monitor the pH in the reaction vessel and automatically adjust the pH using a programmed syringe pump that would dispense NaOH to neutralize the released acid. Figure 1 shows the experimental setup where two eppendorf tubes containing LNPs are lowered into a water bath kept at 37 °C in the centre, flanked by two syringe pumps on either side. The pumps are loaded with syringes containing NaOH and the syringes' needles are pointed into the eppendorf tubes. pH electrode probes are inserted into each of the eppendorf tubes (see Figure 2 for a close up) to monitor the pH values and the pH meter device is off screen. The LNPs were stirred with tiny magnetic stirrers.

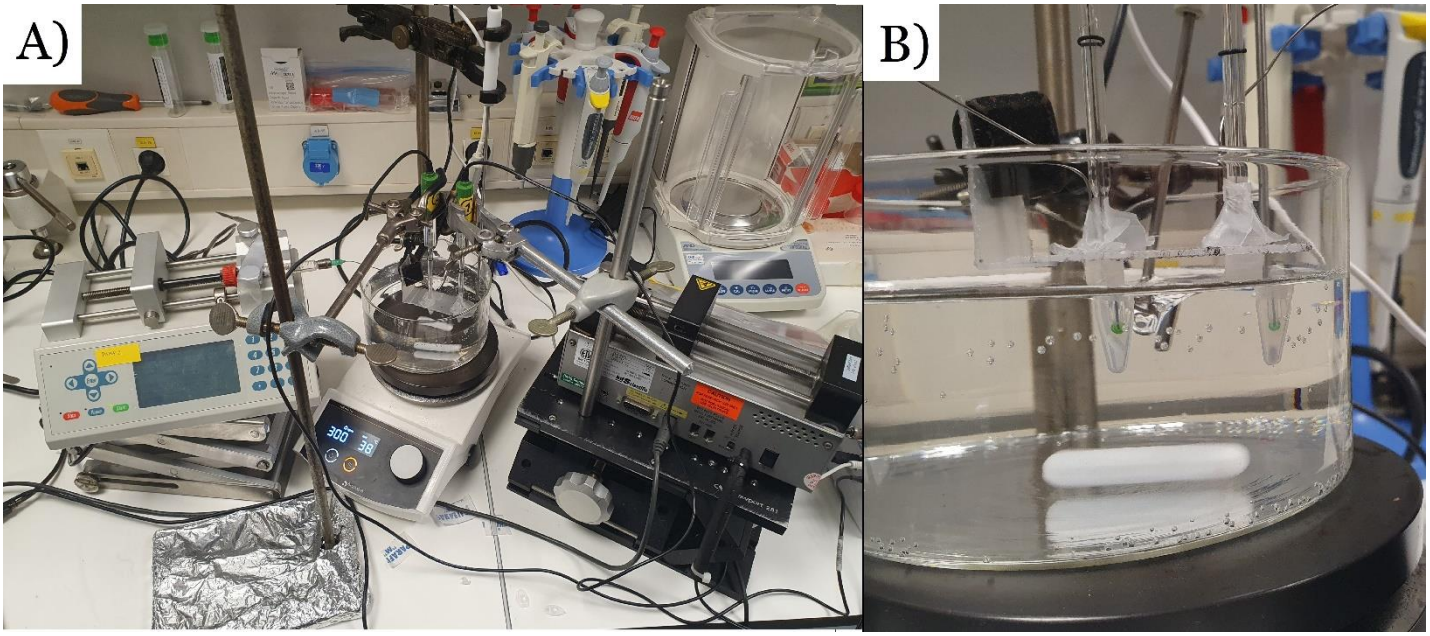

***Figure 1**. A) pH-stat experimental setup, featuring a temperature controlled water bath in the centre that holds the samples and two syringe pumps to either side of it that dispense NaOH to keep the pH of the reaction constant. B) Close up of the eppendorf tubes containing the samples and being monitored by pH electrode probes (with green tips).*

Two flow-through capillaries were put into the capillary holder of the beamline and connected by HPLC tubing to the corresponding eppendorf tubes containing the LNPs. The tubing was fed through a peristaltic pump to continuously feed the LNPs through the capillaries. The capillary holder could then be regularly moved to collect scattering patterns from either of the two capillaries periodically. **Unfortunately, the dead volume within the HPLC tubes was not compatible with the sample volume that could be produced for a reliable SAXS measurement. Due to expensive nature of the siRNA-loaded LNPs and the relatively high concentrations required for a reliable SAXS measurement, only limited volume of the samples could be produced and was unfortunately not sufficient for a viable flow-through experiment.** Regular samples had to be taken from the eppendorf tubes manually and loaded into borosilicate capillaries for static SAXS measurements. For samples that did not degrade very fast, the pH could be monitored and adjusted manually. The samples were kept at 37 °C and continuously mixed using a shaker from the PSCM lab (Figure 2).

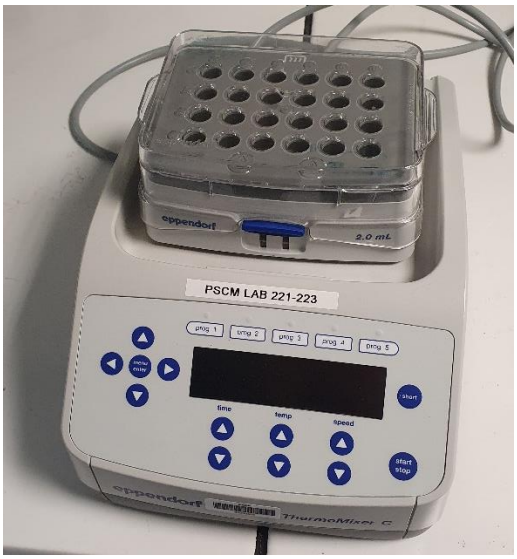

***Figure 2**. Temperature controlled shaker that was used for samples with slow degradation kinetics. pH in the eppendorf tubes was regularly measured and adjusted, if needed. Samples could be taken out for SAXS measurements and then put back into the tubes.*

The scattering setup had a sample-to-detector distance of 2458 mm and wavelength of 1.0332 Å for the SAXS experiments, providing an approximate q range of 0.09 – 4.7 nm^{-1}. Measuring WAXS was not of importance and the position of the detector was optimized. Cappillaries used were 1.5 mm thick.

Results

The **control experiment** contained LNPs made using the commercially available lipid DLin-MC3-DMA (Figure 3) without the presence of any pH-degradable lipid. Regular SAXS measurements were collected over a 24 h period while the pH was kept at 7.4 and are presented in Figure 4. As expected, the samples exhibited a low-*q* upturn in intensity due to the presence of particles with dimensions larger than the experimental resolution of the setup (diameter measured by DLS is around 120 nm, so we presumably see the scattering from their surface at low-*q*). Additionally, a correlation peak could be observed at around $q \sim 1$ nm^{-1}, attributed to the internal arrangement of siRNA within the LNPs. The curves did not exhibit significant changes over time.

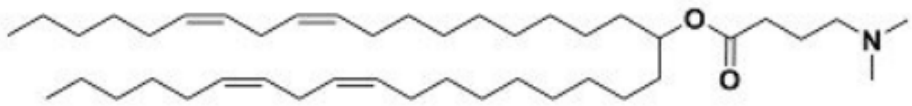

***Figure 3**. Chemical structure of DLin-MC3-DMA lipid.*

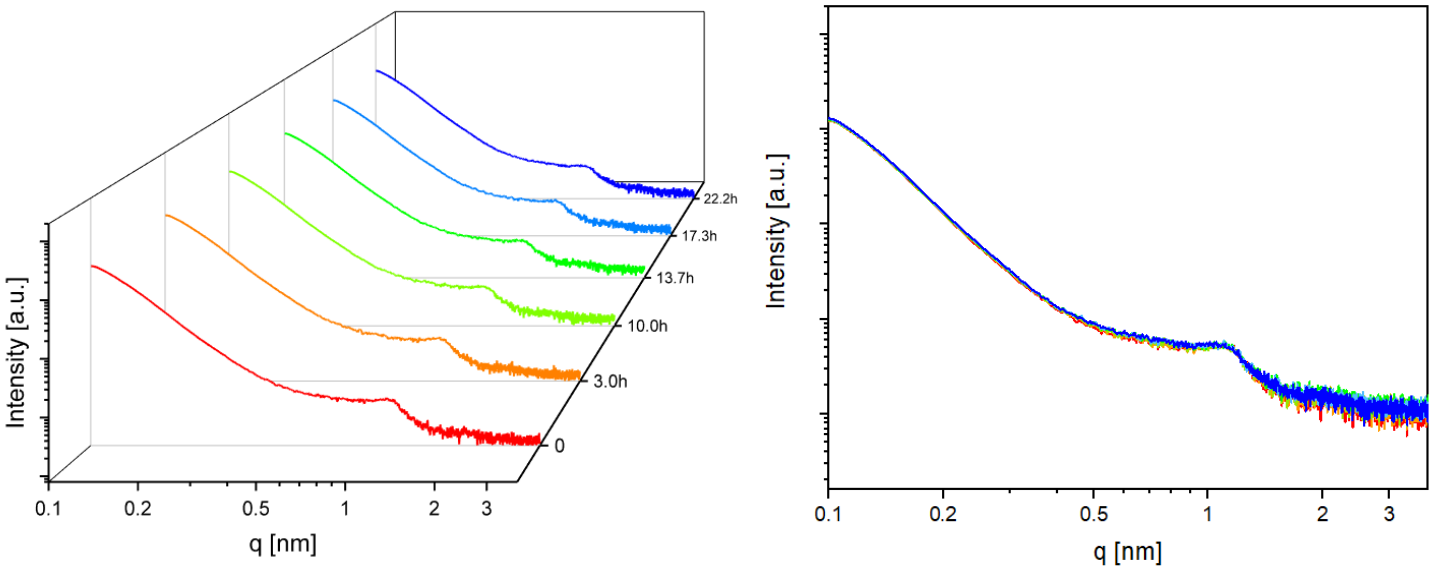

***Figure 4.** SAXS scattering patterns of LNPs without any pH-degradable lipids at pH 7.4.*

In parallel, the same LNPs were subjected to pH 5.5 for 2 h and then had their pH returned back to 7.4. This was done to simulate the pH conditions that could be encountered in the endosome upon uptake of these LNPs by cells. Figure 5 shows the SAXS patterns before the low pH conditions, during the low pH conditions (0h – 2h), and upon return of pH to neutral conditons (pH 7.4, 2h – 24h). Due to changes in the protonation state of the amine group on DLin-MC3-DMA, the LNPs undergo structural rearrangements at low pH. Note that the changes are nearly fully reversible when the pH is returned to 7.4. After pH is returned to 7.4, the structure remains stable for the rest of the experiment.

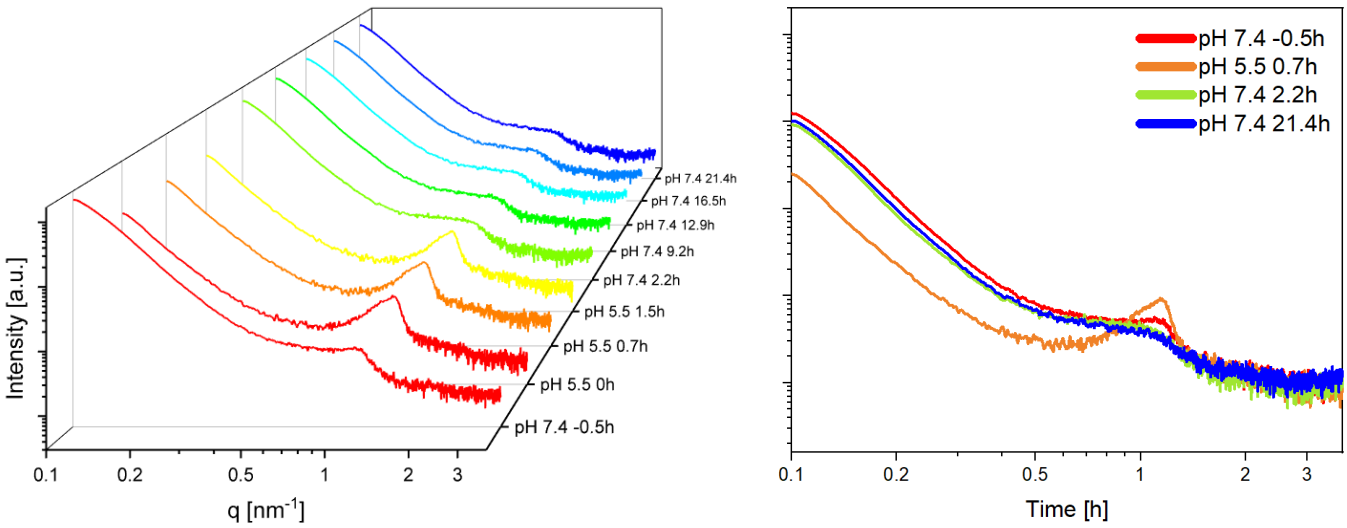

Figure 5. *SAXS scattering patterns of LNPs without any pH-degradable lipids that were subjected to pH 5.5 for 2 h and then returned to pH 7.4.*

When the LNPs with DLin-MC3-DMA were made with the newly synthesized lipid pMeO PABC OA (Figure 6), their nanostructure was observed to gradually change over time when kept at pH 7.4 (Figure 7). The low-q upturn remained, while the correlation peak at $q \sim 1$ nm^{-1} gradually dissapeared over time (Figure 7).

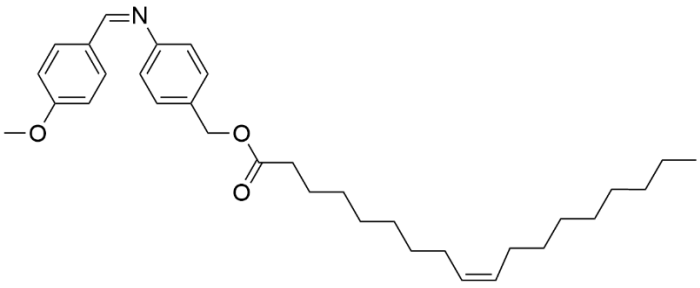

Figure 6. *Chemical structure of pMeO PABC OA lipid.*

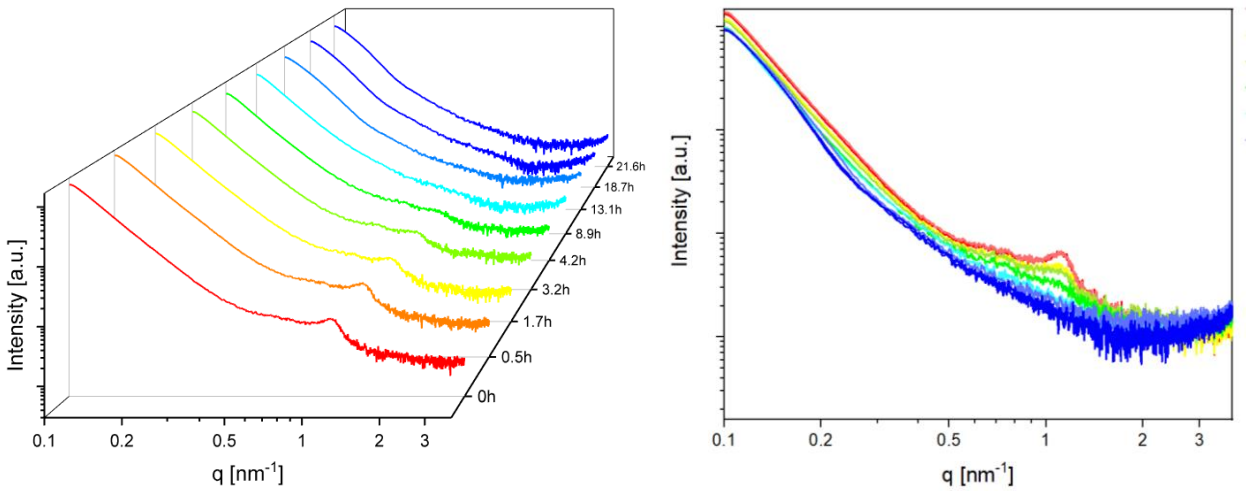

Figure 7. *SAXS scattering patterns of LNPs containing 10 mol% of degradable mMeO PABC OA lipid at pH 7.4 over 24 h period. Loss of correlation peak at $q \sim 1$ nm^{-1} indicates changes in the internal nanostructure of the LNPs.*

When the LNPs containing the degradable mMeO PABC OA lipid were subjected to 2 h at pH 5.5, the correlation peak at $q \sim 1$ nm^{-1} became more pronounced at pH 5.5 and then shifted to lower q values when pH returned to 7.4 (Figure 8). Over time, the correlation peak was observed to shift to even lower q values and gradually diminish in intensity.

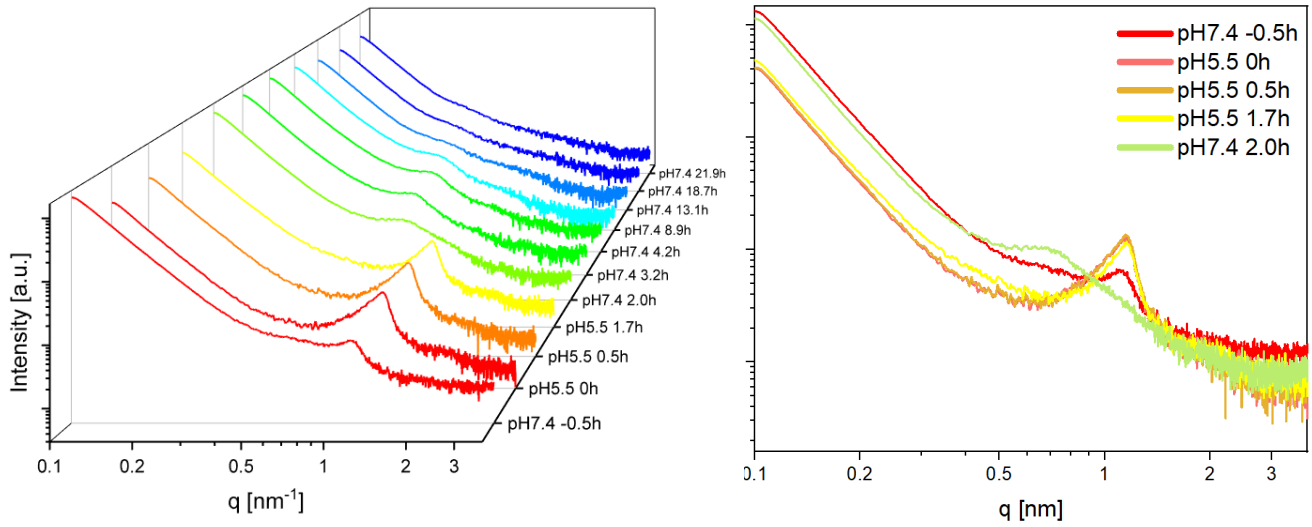

Figure 8. *SAXS scattering patterns of LNPs containing 10 mol% of degradable mMeO PABC OA lipid when subjected to pH 5.5 for 2 h and then returned to pH 7.4. Shifts in the position and intensity of the correlation peak at $q \sim 1$ nm^{-1} indicate changes in the internal nanostructure of the LNPs.*

Most interestingly, if the lipid mMeO PABC OA was modified with an amide bond instead of the imine bond (see Figure 9), the molecule would not undergo pH-triggered degradation. When making LNPs contaning the pH-resistant amide version of mMeO PABC OA, the internal nanostructure of the LNPs appeared to be remain largely intact at pH 7.4 and even when subjected to pH 5.5 for 2 h (Figure 10 and 11).

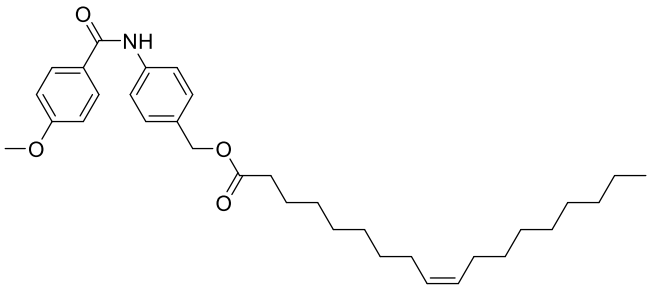

Figure 9. *Chemical structure of pH-resistant amide version of pMeO PABC OA lipid.*

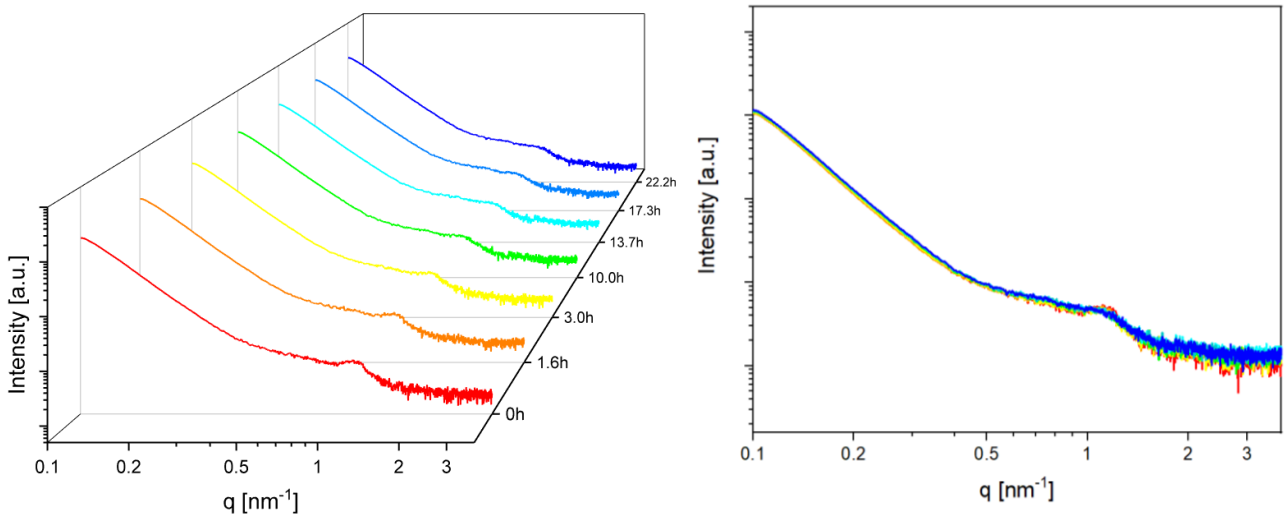

Figure 10. *SAXS scattering patterns of LNPs containing 10 mol% of pH-resistant mMeO PABC OA lipid at pH 7.4 over 24 h period.*

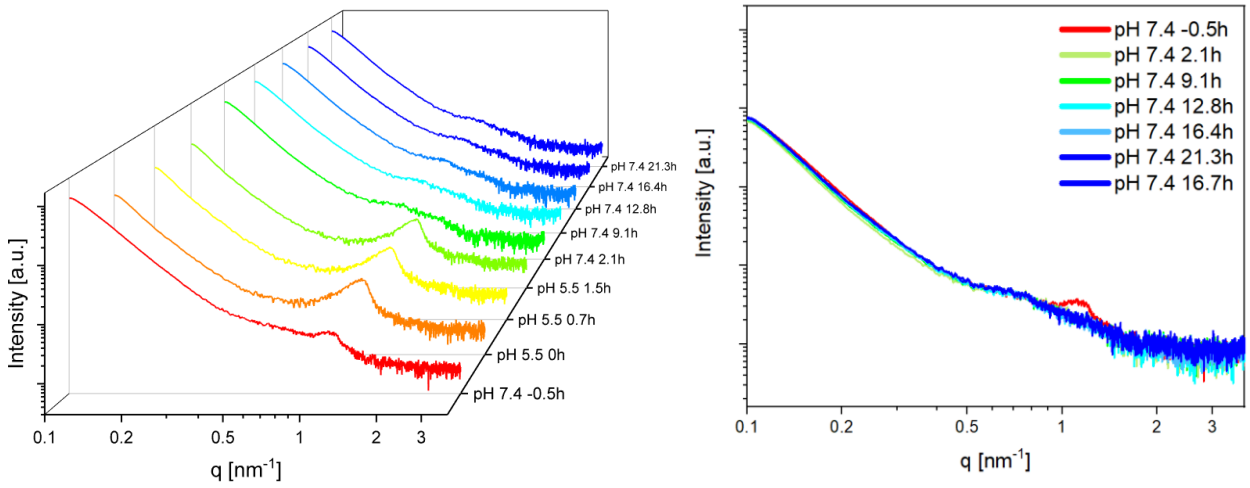

Figure 11. *SAXS scattering patterns of LNPs containing 10 mol% of pH-resistant mMeO PABC OA lipid when subjected to pH 5.5 for 2 h and then returned to pH 7.4. The correlation peak at q ~ 1 nm^{-1} was observed to diminish in intensity after the pH changes.*

Similar data has been collected for a multitude of lipids, shown in Figure 12. For lipids containing an ionizable tertriary amine group, the LNPs were created by replacing the DLin-MC3-DMA lipid with the newly synthesized one.

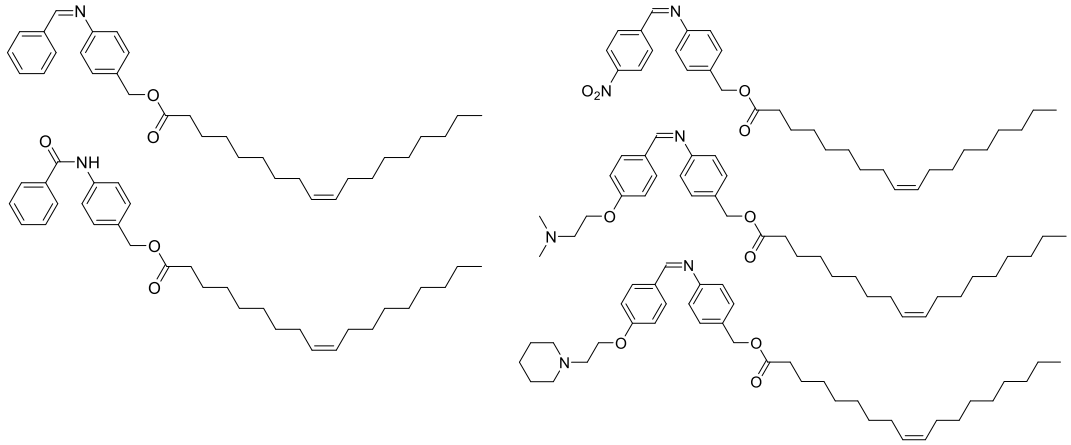

Figure 12. *Chemical structure library of lipids used in the degradation study.*

Additionaly experiments were carried out using lipids that were functionalized with drug molecules IMDQ and STING at the of their PEG tails (Figure 13). These lipids were formulated into LNPs using HSPC and DSPE as a helper lipids (Figure 13). The resulting SAXS patterns (see Figure 14) could not be fitted with a sphere model or a core-shell bicelle model. Instead, a two-shell bicelle model from *Yang et. al., Langmuir 2019, 35, 9483-9492* had to be used to account for the additional PEG layer that contained the drug molecules. The model code has been implemeted with IgorPro and fitting is currently on the way.

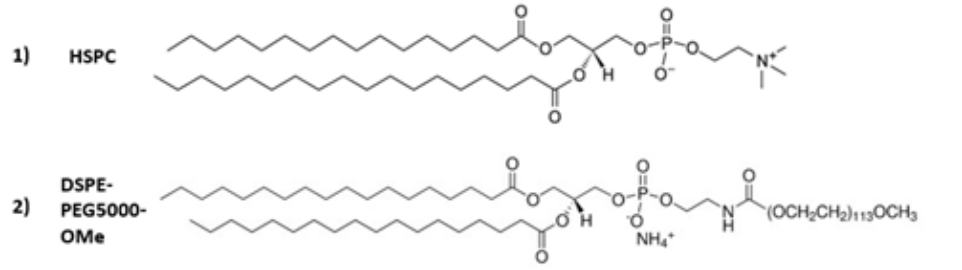

Figure 13*. Chemical structures of lipids used to make core-shell bicelles. The IMDQ and STING conjugated lipids have been ommited for confidentiality.*

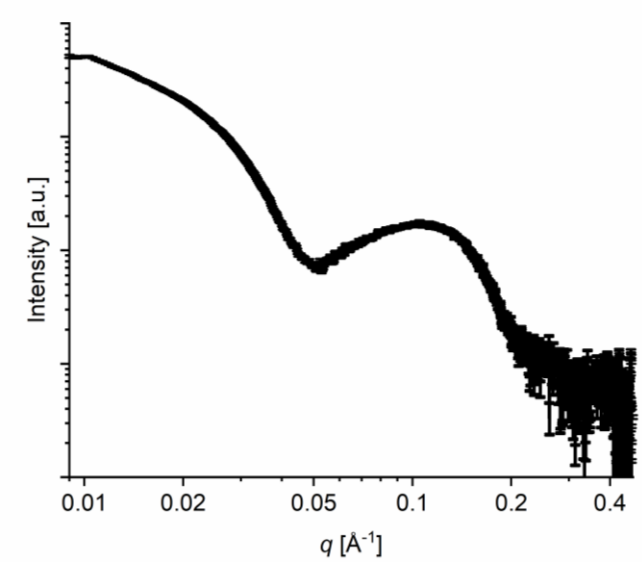

Figure 14. *Representative SAXS pattern of LNPs formed with HSPC and the PEGylated lipids. No pH-triggered degradation was observed.*

Conclusion

pH-triggered colloidal transformations were observed as a function of pH and time. By varying the lipid composition, the kinetics of these transformations could be tuned. Namely, chemical modifications to the lipids that would stabilize the imine bond (by converting it to an amide bond, or by changing the substituents on the benzene right (different variants listed in Figure 12)) were found to influence how fast the internal nanostructure would rearrange itself.

We only have one picture of us from the beamtime. 3 days of beamtime for 2 people was very intense. Yi on the left, Mark on the right, SAXS in the centre.