Method Development for Structural Assessment of Nanolipoprotein Particles with and without Cross-linked Lipids

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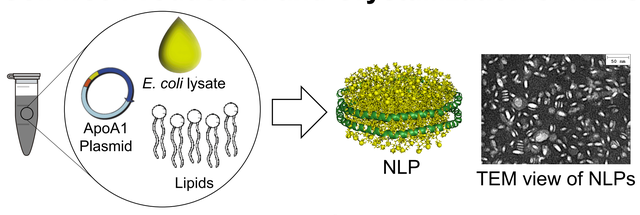
Abstract

Membrane proteins make up approximately 30% of the cellular proteome and account for over 60% of pharmaceutical targets.[1] Determining the structures of this class of proteins is critical to our understanding of disease states and will advance rational drug design. But membrane proteins have limited solubility, rarely form large crystals that diffract well, and often misfold outside of a bilayer, hindering crystallographic studies.[1] Nanolipoprotein particles (NLPs) have arisen as a platform to readily solubilize membrane proteins while mimicking a native lipid environment. NLPs consist of a discoidal phospholipid bilayer encircled by an apolipoprotein belt. In an effort to optimize and improve crystallization of empty NLPs, we altered the fluidity of the lipid bilayer by incorporating photoactive DiynePC phospholipids in the lipid bilayer, forming cross-linked nanoparticles (X-NLPs). Here, we used a cell-free expression system with apolipoprotein A1 (ApoA1) plasmid and micellar lipids to assemble NLPs. Based on high throughput crystallization screening data, we reproduced and validated crystallization conditions for X-NLPs and optimized conditions for non-crosslinked NLPs (DMPC NLPs).

Approach

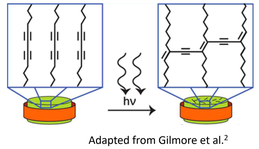
Cell-free Production and Crystallization of NLPs



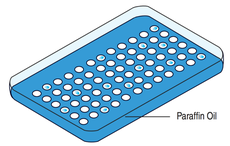
DMPC Lipid:

DiynePC Lipid:

NLPs self-assemble when the Apo1 belt protein is expressed in the presence of lipid vesicles via a cell-free expression system. X-NLPs are produced using an 80:20 DMPC:DiynePC lipid mixture and are then exposed to UV to cross-link the lipids.



Adapted from Gilmore et al.[2]



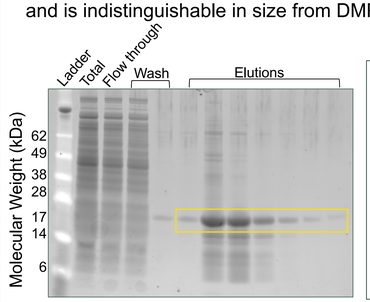
Microbatch plate (under oil)

Crystallization screens were performed using vapor diffusion and microbatch plates. These methods allow for slow, controlled crystal growth.

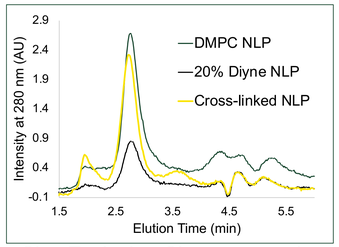
Results

NLP Purification and Characterization

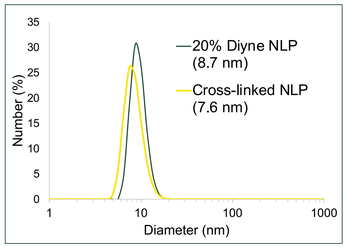
The NLP belt protein ApoA1 contains a 6xHis-tag. Approximately 1.6 mg of 20% Diyne NLPs were produced by cell free expression and purified by Ni affinity. This yielded a population of X-NLPs that is mostly homogenous in size and is indistinguishable in size from DMPC NLPs, as characterized by SEC and DLS.



SDS-PAGE after Ni Purification of 20% Diyne NLPs



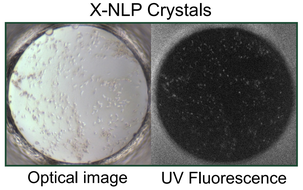
Size Exclusion Chromatography: DMPC NLPs, 20% Diyne NLPs (before crosslinking), and X-NLPs



Dynamic Light Scattering: 20% Diyne NLPs (before crosslinking), and X-NLPs

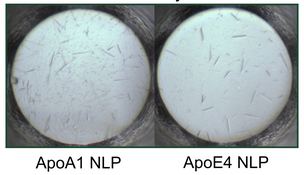
Analysis of High-Throughput Crystallization Screens

X-NLP Crystals



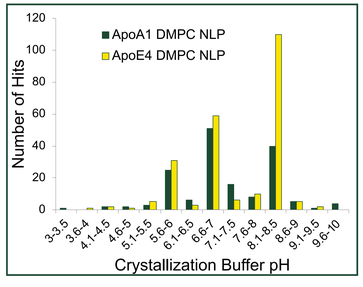
Optical image UV Fluorescence

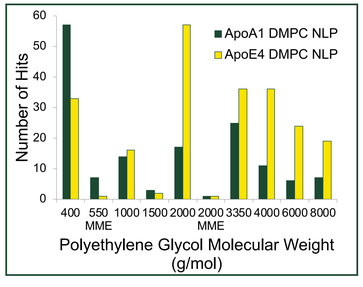
DMPC NLP Crystals



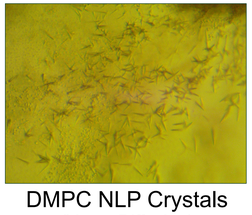
ApoA1 NLP ApoE4 NLP

Chemical conditions of crystallization hits from high throughput screens were analyzed to determine which conditions were optimal for crystal formation. These conditions include parameters such as buffer, pH, salt composition, and polyethylene glycol (PEG) molecular weight and concentration.

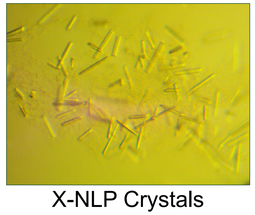




Validation and Optimization of Crystallization Conditions at LLNL



DMPC NLP Crystals (Vapor Diffusion)



X-NLP Crystals (Microbatch)

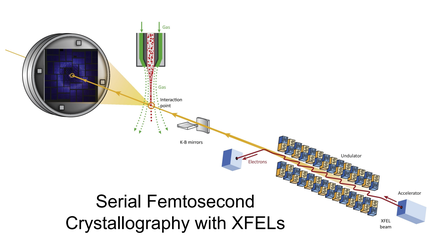
Crystallization conditions of interest were selected, each containing salt, buffer, and polyethylene glycol. These conditions were screened on vapor diffusion and microbatch plates. DMPC NLP crystals were observed after 5 days, and X-NLP crystals were observed after 12 days. X-NLP were successfully crystallized using 0.1 M potassium carbonate, 0.1 M CAPS buffer pH 10, and 39% (w/v) PEG 4000. X-NLP crystals were approximately 70 µm long.

Conclusion

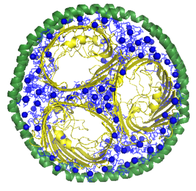
I was able to produce mg-scale amounts of purified X-NLPs through cell-free expression and UV cross-linking, and I characterized their size distribution and confirmed nanoparticle formation by size exclusion chromatography and dynamic light scattering. I also identified chemical conditions that promote DMPC NLP and X-NLP crystal growth and subsequently validated those conditions for X-NLPs and optimized conditions for DMPC NLPs at LLNL. The X-NLP crystals are significantly larger and more uniform than those of the noncross-linked NLPs, suggesting that they may have superior diffraction quality for X-ray crystallography. This work has **advanced our current knowledge of X-NLPs and improved methods for readily crystallizing NLPs.** This work also represents the **first instance of verified crystallization of cross-linked NLPs.**

Future Directions

The methods developed in this study will be used to grow microcrystals of DMPC NLPs and X-NLPs for use in serial femtosecond crystallography using XFELs. X-NLPs may have improved diffraction quality compared to DMPC NLPs, which could afford more exact structural determinations of empty NLPs. Future researchers can build off of this study to optimize conditions for crystallizing membrane proteins embedded in NLPs. This will open up possibilities for structural studies of membrane proteins with potentially exciting therapeutic applications, such as the chlamydial major outer membrane protein (MOMP), which is a promising candidate for a chlamydia vaccine.



Serial Femtosecond Crystallography with XFELs



MOMP in an NLP*

*Artistic representation. PDB: 3POX

References and Acknowledgements

[1]Shelby, Megan L. et al. *Front. Pharmacol.* **2019**, 10. 744.
[2]Gilmore, Sean et al. *ACS Appl. Mater. Interfaces.* **2016**, 8, 20549−20557

High-throughput crystallization screens were performed by Hauptman-Woodward Medical Research Institute and analyzed at LLNL. This work was supported by funds from the following: National Institute of Health: NIGMS grant R01GM117342 and NIAID grant R21AI120925. This work was performed under the auspices of the U.S. Department of Energy by Lawrence Livermore National Laboratory under Contract DE-AC52-07NA27344.

The 2019 STEM Teacher and Researcher Program and this project have been made possible through support from Chevron (www.chevron.com), the National Marine Sanctuary Foundation (www.marinesanctuary.org), the National Science Foundation through the Robert Noyce Program under Grant #1836335 and 1340110, the California State University Office of the Chancellor, and California Polytechnic State University in partnership with Lawrence Livermore National Laboratory and University of Oregon Noyce ESPRIT, funded by NSF DUE 1660724. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the funders.

LLNL-POST-782417