Development of an Integrated Analytical Workflow for Characterization of Athebody® DARPin Targeted Lipid Nanoparticles

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Abstract

Background and Objectives

Extrahepatic delivery of lipid nanoparticle (LNP) therapeutics has emerged as an important area of research for advancing nucleic acid medicines. While current LNP technologies have demonstrated effective hepatic delivery, achieving efficient delivery to extrahepatic tissues remains challenging and is critical for broadening the application of nucleic acid therapeutics. Recent advances, including our demonstration of Athebody® DARPin (Designed Ankyrin Repeat Proteins)-mediated targeted LNP delivery to extrahepatic cells in collaboration with Athebio, underscore the need for analytical methods to characterize these targeted LNP. Here, we describe the development of analytical methods to support the characterization of DARPin-targeted LNP.

Materials and Methods

DARPin-targeted LNP were prepared by post-insertion of a DARPin-PEG Lipid into pre-formed LNP. A complimentary panel of analytical methods was developed to support in-process and final product characterization. UPLC-SEC was used to assess DARPin purity and quantify the total DARPin–PEG Lipid content, while the BCA assay was used to quantify protein content. UPLC-RP was used to evaluate ligand conjugation and quantify lipid content. DLS and cryo-EM were used to characterize particle size and morphology, respectively. Total RNA content was measured by ion-pairing UPLC-RP, while UPLC-AEX was used to assess unencapsulated RNA. Together, these methods provide a comprehensive approach for monitoring critical quality attributes throughout targeted LNP manufacturing and assessing final product quality.

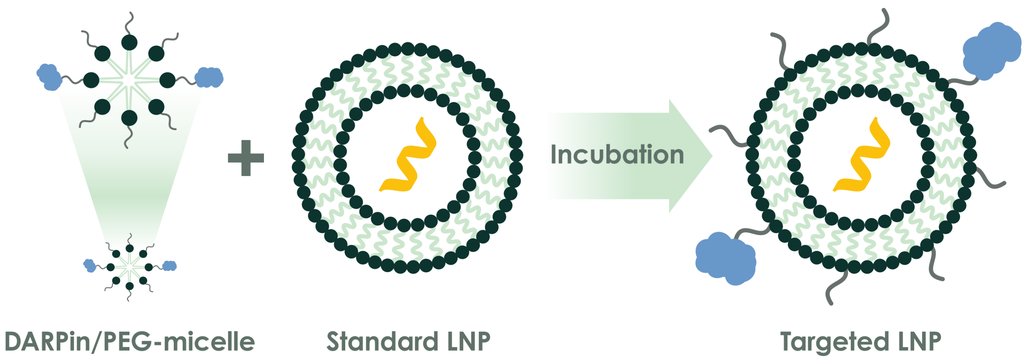
1 Athebody® DARPin & LNP association process

A



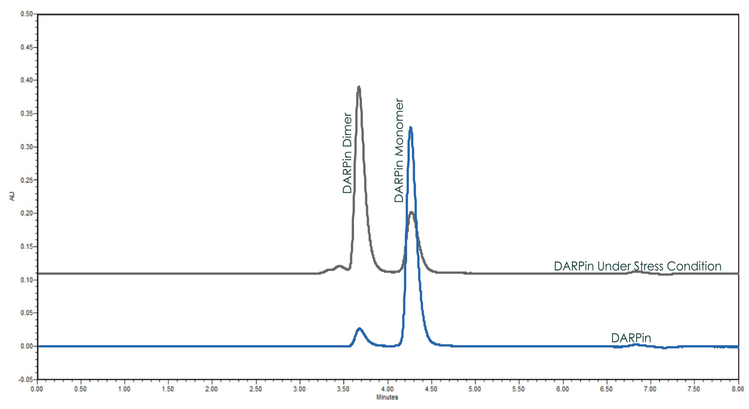
- **Very stable monomeric protein (15 – 20 kDa) with no disulfide bonds**
- **Allows for site-specific conjugation via an engineered cysteine amino acid to a maleimide-containing PEG-lipid**

B



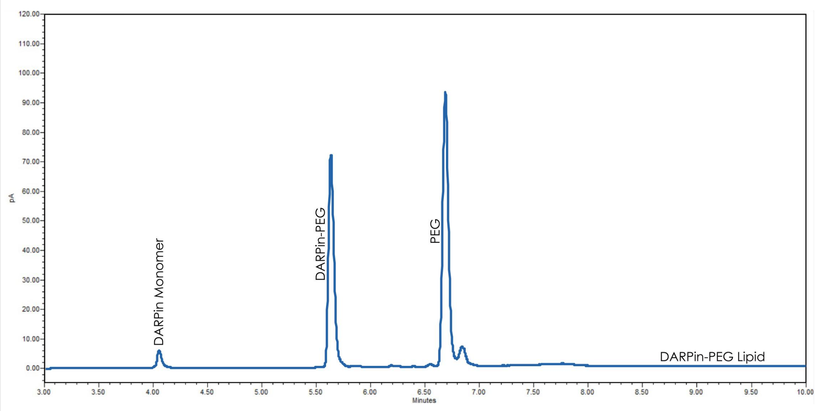
(A) DARPins are a class of antibody mimetics composed of two caps (N-cap and C-cap) and 2-4 library modules that contain the variable (target binding) region. **(B)** The LNP used in these studies are composed of a Ionizable lipid, distearoylphosphocholine (DSPC), cholesterol and a PEG-lipid. DARPin-LNP were made by insertion of a DARPin-PEG-lipid conjugate to the LNP.

2 DARPin Purity



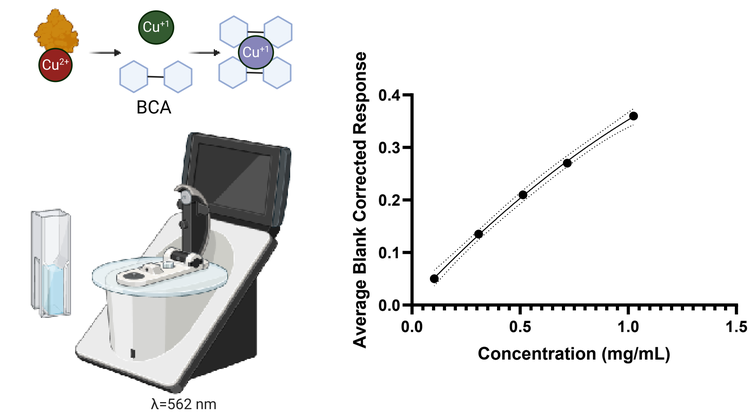
Athebody® DARPin purity is assessed using Ultra-Performance Liquid Chromatography–Size Exclusion Chromatography (UPLC-SEC) with an ultraviolet (UV) detector. Size Exclusion Chromatography (SEC) separates molecules based on size, with larger molecules eluting earlier than smaller molecules due to differences in their access to the pores of the stationary phase. This method is used to evaluate DARPin raw material purity and monitor stability and degradation, including dimer formation and other higher-molecular-weight species.

3 Ligand Conjugation Efficiency



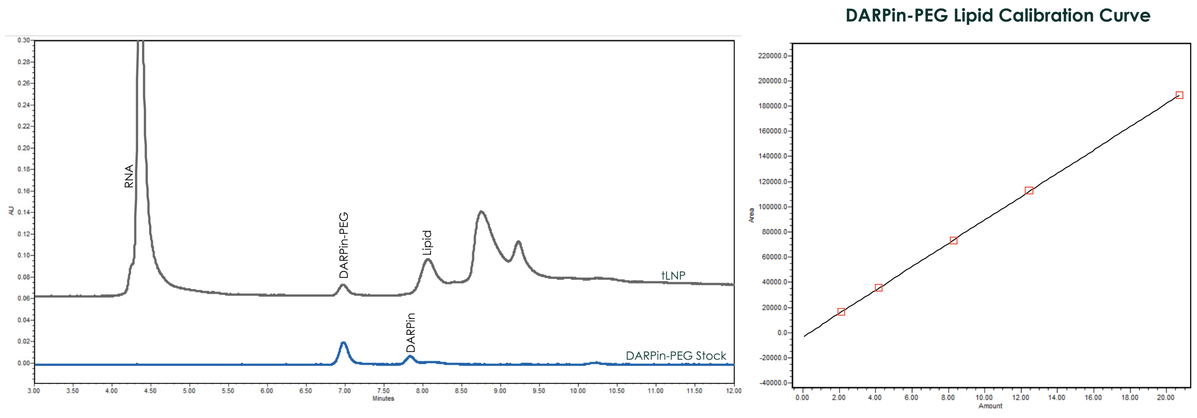
DARPin-PEG conjugation efficiency is analyzed by Ultra-Performance Liquid Chromatography–Reverse Phase Chromatography (UPLC-RP) with a Charged Aerosol Detector (CAD). Reverse Phase Chromatography (RP) separates molecules based on hydrophobicity, with more hydrophobic molecules generally retained longer by the nonpolar stationary phase. This enables accurate determination of conjugation efficiency of the DARPin-Maleimide reaction due to the excellent separation between DARPin monomer and DARPin-PEG lipid.

4 DARPin Quantitation



DARPin content is measured using the bicinchoninic acid (BCA) assay, a colorimetric method that determines protein concentration through a reaction between proteins and copper ions, producing a purple-colored complex. The intensity of the color is proportional to the protein concentration in the sample. The absorbance is measured at 562 nm using a spectrophotometer, and the DARPin concentration is determined by comparing the measured absorbance with a calibration curve generated using standards of known DARPin concentrations.

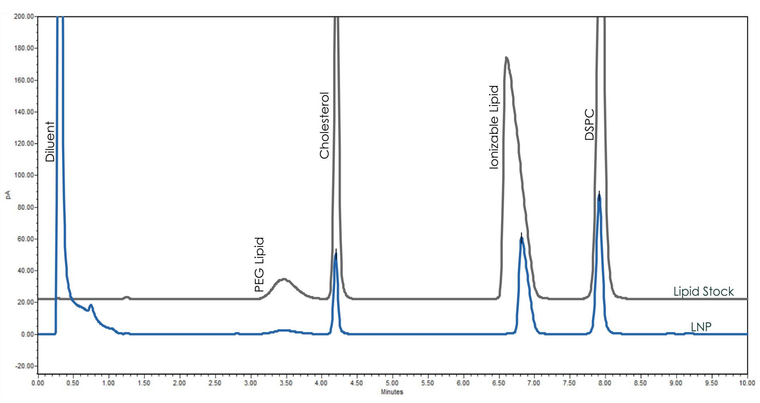
5 DARPin-PEG Lipid Quantitation Assay



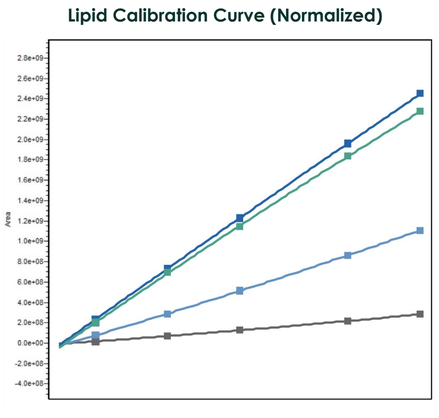
DARPin-PEG Lipid Calibration Curve

Total DARPin-PEG lipid content in targeting lipid nanoparticles (tLNP) is quantified using a UPLC-SEC-UV method. Diluted samples are analyzed against a calibration curve prepared with known concentrations of DARPin-PEG lipid. The nanoparticles are disrupted using a mobile phase containing a low concentration of detergent to enable ligand quantitation. While primarily intended for final-product analysis, the method can also be applied to other purposes and, in combination with complementary analytical techniques, can help assess other relevant process parameters such as ligand insertion and binding efficiency.

6 Lipid Quantitation & Composition

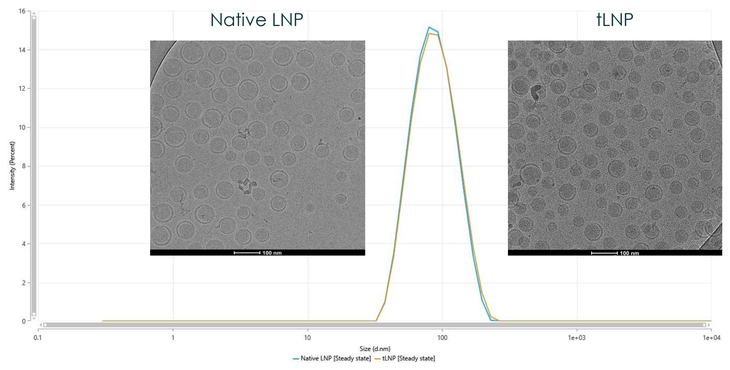


Lipid Calibration Curve (Normalized)



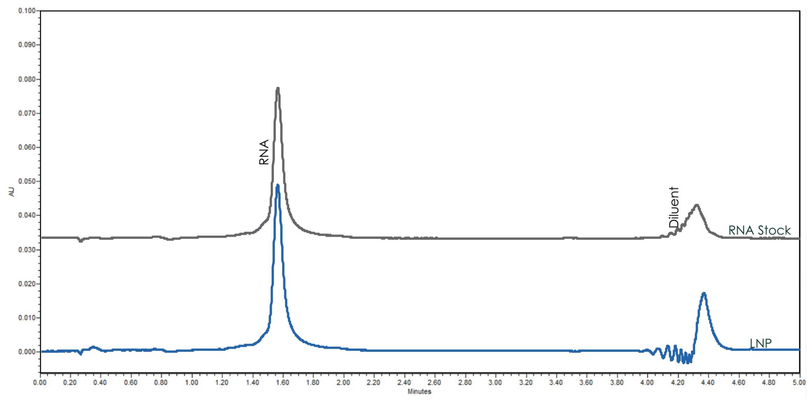
Total lipid content and relative lipid composition of tLNP are determined using a UPLC-RP-CAD method. Diluted samples are analyzed against calibration curves prepared from lipid mixtures of known concentrations and ratios. Individual lipid concentrations are quantified using the corresponding calibration curves, and the relative composition is calculated from these values. The method can also be used for in-process quality control to confirm lipid concentrations and composition in intermediate samples.

7 LNP Size & Polydispersity Index



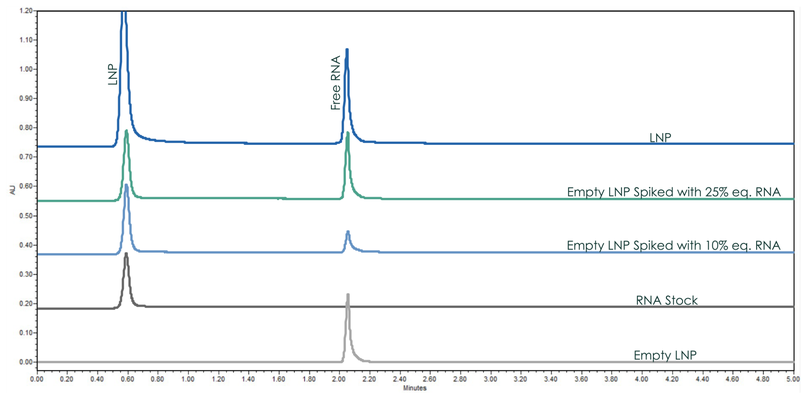
The LNP average size and polydispersity index (PDI) are determined using dynamic light scattering (DLS). DLS measures fluctuations in scattered light caused by the Brownian motion of particles to estimate their hydrodynamic diameter and size distribution. Alternatively, cryo-electron microscopy (cryo-EM) can be used to assess the size and morphology of LNP by directly visualizing the particles in a vitrified state.

8 RNA Quantification & Adduct



Total RNA content in LNP samples are measured using a UPLC-RP-UV method with an ion-pairing reagent (iPRP). Samples are diluted with a detergent-containing disruption buffer and analyzed against a calibration curve or single-point calibration prepared from RNA stock of known concentration. Lipid-adducted RNA can also be detected and quantified using this assay, with the percentage of lipid adduct calculated based on its peak area relative to the total RNA peak area. The method is applicable to final-product analysis, in-process monitoring, and stability studies.

9 Free RNA Quantification & RNA Encapsulation



Free RNA Content in LNP is assessed by an Ultra-Performance Liquid Chromatography–Anion Exchange Chromatography (UPLC-AEX) method. AEX separates molecules based on differences in net negative charge through electrostatic interactions with a positively charged stationary phase, resulting in distinct elution profiles. With an appropriate elution buffer, negatively charged free RNA can be effectively separated from neutral LNP, enabling quantification of free RNA and assessment of RNA encapsulation efficiency.

Summary

1. A comprehensive set of analytical methods was established to assess the quality and characteristics of DARPin-targeted LNPs.
2. These methods support monitoring during manufacturing and help ensure the quality of the final product.

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