# Formulation methods for peptide-modified lipid nanoparticles

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**Statement of Purpose:** Lipid nanoparticles (LNPs) are currently the most clinically advanced non-viral gene therapy carrier and potential applications are abundant. However, there is an outstanding need to develop LNPs for cell specific delivery in extrahepatic organs. Peptides are potentially attractive ligands because they are relatively inexpensive to synthesize and are more stable, but can still represent a large diversity of bioactive binding motifs. Several studies have demonstrated the potential for peptides to improve LNP targeting to specific cell populations, but no study has yet compared different methods of peptide conjugation or the effects of different sizes of peptides on LNPs and their resulting physicochemical properties and ability to target cells. In this study, our goal was to compare two methods for the incorporation of peptides into LNP formulations: (1) post-conjugation targeted (PCT) formulation, in which we formulated LNPs with maleimide handles on the PEG-lipid that were subsequently modified with peptide post-formulation, and (2) in-line targeted (ILT) formulation, in which we synthesized peptide-PEG-lipid conjugates that were directly used for LNP formulation.

**Methods:** LNPs were formulated via a microfluidic mixer, with lipids dissolved in ethanol in one channel and RNA dissolved in an acetate buffer in the other channel. The physicochemical properties of these LNPs—hydrodynamic diameter, polydispersity index, and zeta potential—were measured. We then compared the cellular interaction of the in-line and post-conjugation targeted LNPs. PCT and ILT LNPs labeled with cyclic RGD, a peptide known to bind to integrins, were incubated with cancer cells that overexpress integrins. Binding at 4°C and uptake at 37°C were quantified via flow cytometry, and transfection efficiency of peptide-labeled FLuc mRNA LNPs was quantified via a luciferase assay. The organ-level biodistribution and transfection efficiency *in vivo* of PCT and ILT LNPs was measured via DiR labeling and luciferase assay, and cell tropism was evaluated via Ai9 transgenic mice (Jackson Labs, Bar Harbor, ME) and Cre mRNA LNPs.

**Results:** We compared the physicochemical properties of LNPs formulated with each method and found that LNPs had similar sizes, zeta potentials, and peptide content for peptides of various lengths, with the exception of ILT LNPs that aggregated when formulated with a large peptide. Using cyclic RGD LNPs as a model, we compared the binding, uptake, and transfection efficiency PCT and ILT LNPs, observing that while binding and uptake were similar, cells treated with PCT LNPs expressed more luciferase (Fig. 1) and that PCT LNP formulations had higher yields of RNA in the final solution relative to the RNA input. The biodistribution and transfection efficiency in each organ of ILT and PCT LNPs was measured via DiR labeling and luciferase assay. PCT and ILT LNPs had similar, decreased blood half-lives compared to untargeted LNPs. PCT and ILT LNPs also had similar organ biodistribution, accumulating more in the spleen and lungs compared to untargeted LNPs, but PCT LNPs demonstrated slightly greater transfection than ILT LNPs in all organs except the kidney. Via Ai9 mice expressing tdTomato in cells exposed to Cre and Cre mRNA LNPs, we investigated cell tropism of PCT cRGD LNPs compared to untargeted LNPs and observed that cRGD targeting increased transfection of endothelial cells (Fig. 2). In particular, cRGD LNPs in the heart appeared to show a shift in tropism from cardiomyocytes to endothelial cells.

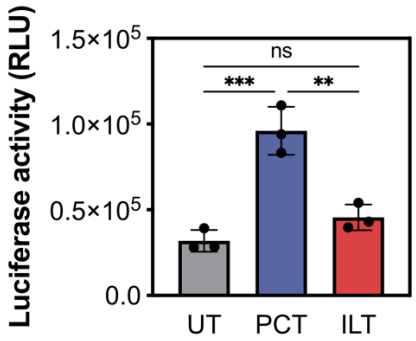


Fig. 1: PCT LNPs mediate greater luciferase activity than ILT LNPs or untargeted (UT) LNPs *in vitro*

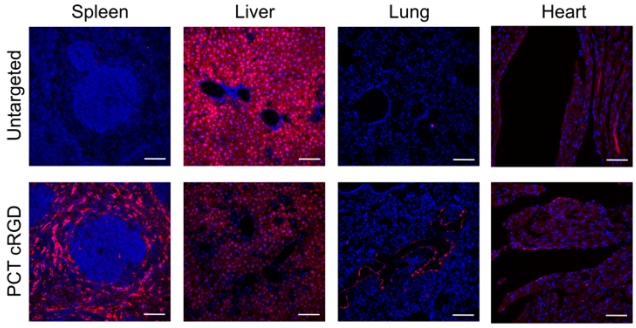


Fig. 2: PCT cRGD LNPs mediated increased transfection in endothelial cells in several organ types. (Blue, DAPI; red, tdTomato)

**Conclusions:** In this study, we showed that while different methods for peptide incorporation into LNPs can yield nanoparticles with similar physicochemical properties and cell association, the method of peptide incorporation impacts LNP functionality. Despite the advantages of increased simplicity and control of the in-line formulation process that make it appealing for manufacturing, post-conjugation was found to be a superior process for formulating peptide targeted LNPs because it can incorporate a larger diversity of peptides, and forms more stable nanoparticles. Furthermore, post-conjugation led to LNPs that were more active *in vitro* and *in vivo*. It is likely that each peptide ligand requires individual optimization of parameters such as conjugation density and linker length. Future studies could investigate a diversity of PEG-lipids used to anchor peptides to the LNP surface.