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Refining peptide-functionalized LNPs for enhanced tumor-targeted mRNA delivery

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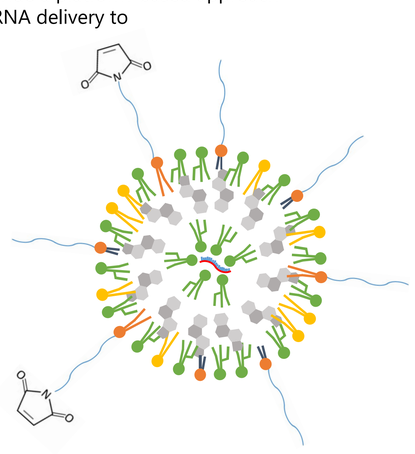
Active targeting with peptide-functionalized LNPs

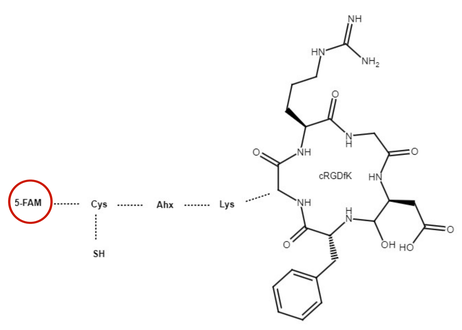
With the advancement of RNA therapeutics - in this context regarding LNPs - one of the key challenges remaining is the need for tissue specific extrahepatic targeting strategies. Our aim is to provide a platform-based approach for peptide-functionalized LNPs utilizing active targeting to achieve selective mRNA delivery to tumor tissue-associated endothelial cells after intravenous application.

	mol%
Lipid 5	50.0
Cholesterol	38.3
DSPC	10.0
DSPE-PEG$_{2000}$	1.0
DSPE-PEG$_{2000}$ maleimide	0.5

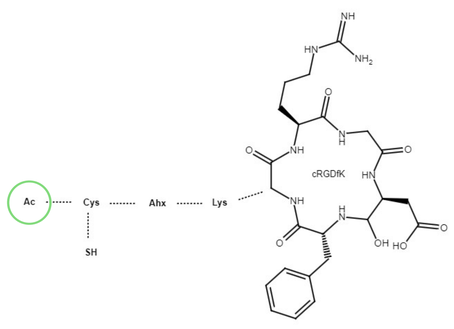


RGD peptides as targeting ligands

For this purpose, RGD peptides - which are prototypic ligands for the αvß3 integrin receptor, that is overexpressed on various tumor and endothelial cells - are used as model peptides.



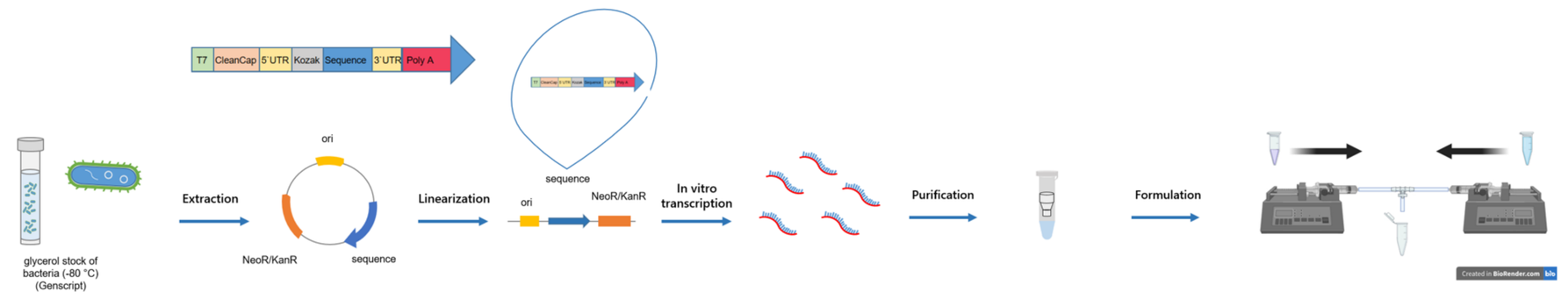
5-FAM-Cys-cRGD



Ac-Cys-cRGD

Methods

From plasmid extraction to in vitro transcription



bacterial Culture | plasmid | linearized DNA sequence | mRNA | column purification | LNP formulation with T-tube setup

LNP Formulation
T-Tube Setup
Dialysis
Ultracentrifugation

Particle characterization
DLS
Cholesterol Assay
Ribogreen assay

Custom mRNA library

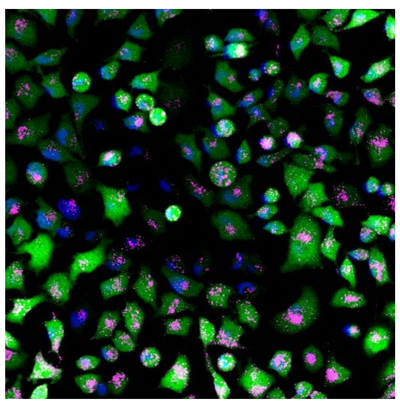
eGFP	established
Fluc	in process
Nluc	in process
Antares	in process
TNFa	established
IL23	available

custom mRNA library

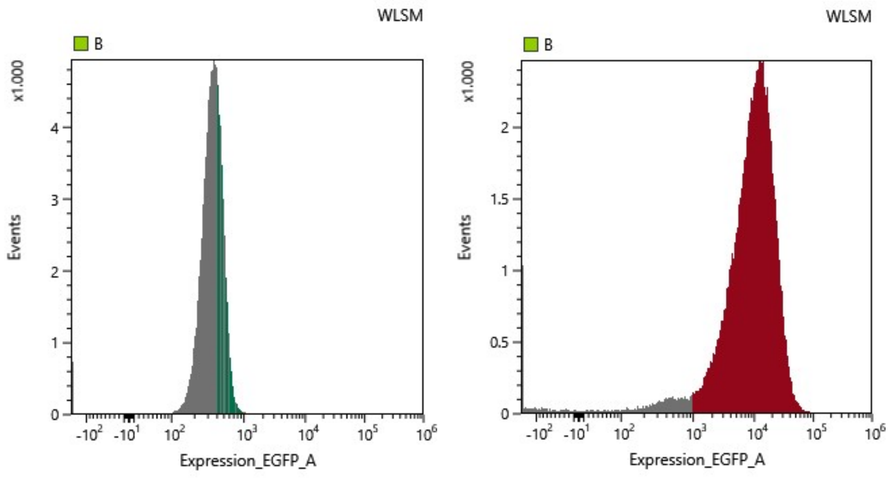
Results

Transfection experiments with LNPs using in-house eGFP mRNA

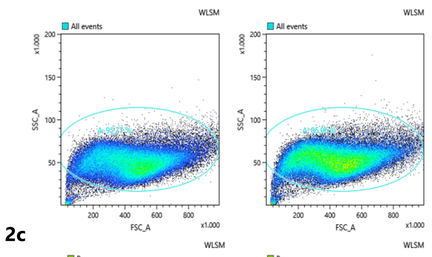
1 TPLSM
HeLa Cells with eGFP LNPs



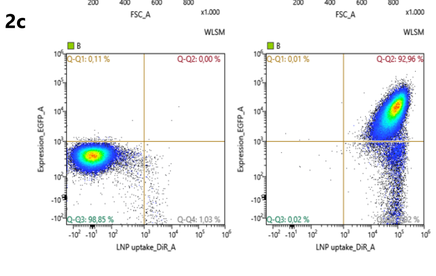
2a 4T1 cells eGFP expression profile in flow cytometry



2b Gating of 4T1 cells



2c



2d eGFP expression in 4T1 cells

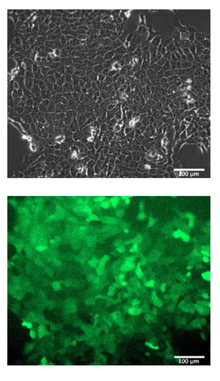


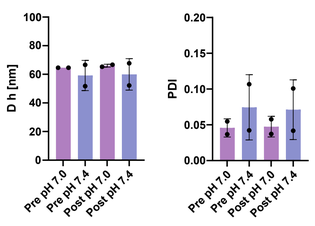
Figure 1 Max Projection of Two-Photon-Laser-Scanning Microscopy (TPLSM) data for HeLa cells transfected with DiR-labeled (0.2 mol%) LNPs with 1 µg in-house eGFP mRNA (N/P ratio =10). The Figure shows eGFP expression (green), a nucleus staining with Hoechst dye (blue) and DiR uptake (pink).

Figure 2 Preliminary FACS data (n=1) for the transfection 4T1 breast cancer cells (100k events) as a suitable tumor model for active targeting studies for in vivo purposes. DiR-labeled (0.2 mol%) LNPs (Lipid 5/cholesterol/DSPC/DMG-PEG2000/DiR 50.0/38.3/10.0/1.5/0.2) with in-house eGFP mRNA (N/P ratio = 10) were used to transfect 4T1 cells. 4T1 cells were seeded at a density of 300k per well in a 6 well plate and transfected with 3 µg of mRNA LNPs after 24h. After 48 hours the cells were trypsinized, washed and suspended in 200 µL of FACS buffer for measurements. Figure 2a demonstrates a clear increase in events with a higher fluorescence intensity in the eGFP channel suggesting a successful transfection. Figure 2b shows the included cell population for the untreated (left) and treated (right) sample. Figure 2c compares the detected fluorescence intensity of DiR (x) to the overall eGFP expression (y) of the cell population. The shift from the lower left quadrant with low detected signals for the untreated sample to the upper right quadrant for the transfected sample indicates that successful cell uptake (DiR) coincides with an increased eGFP expression. Figure 2d depicts a picture taken for the transfected 4T1 cells to illustrate the aforementioned data set.

Parameter changes of LNPs during conjugation protocol

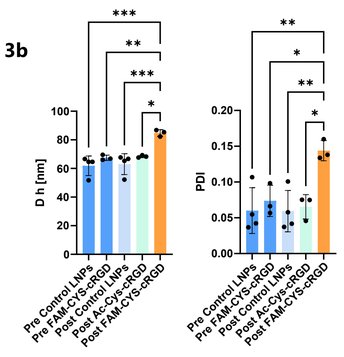
Buffer treatment
HEPES (pH 7.0) or PBS (pH 7.4)

3a



Peptide modification LNPs

3b



Encapsulation efficiency
Peptide-modified LNPs

3c

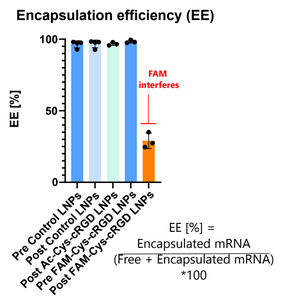
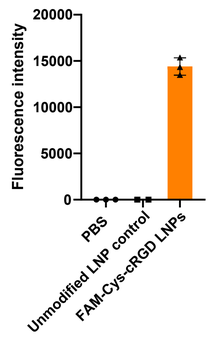


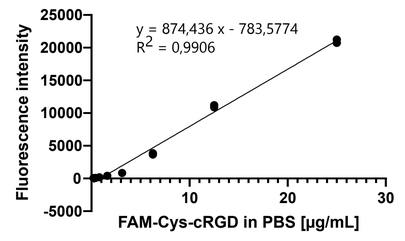
Figure 3 Comparison of LNPs Pre and Post peptide conjugation treatment regarding hydrodynamic Diameter (D h) and Polydispersity Index (PDI) for Dynamic Light Scattering (DLS) measurements as well as Encapsulation efficiency (EE) of in-house eGFP mRNA calculated based on a Ribogreen Assay. Figure 3a illustrates that the variation of the buffer choice between HEPES (pH 7.0) and PBS (pH 7.4) and subsequent centrifugal centrifugation (Pre and Post) of the formulated LNPs does not show to have any visible impact on the chosen parameters of the aforementioned. Figure 3b compares formulated LNPs prior to an in situ maleimide-thiol conjugation step with peptide modified LNPs with and with FAM as a fluorophore label attached to the chosen cRGD peptide for peptide quantification. LNPs with a fluorophore labeled peptide modification show significant differences in hydrodynamic diameter and PDI potentially hinting at the unusability of this method to properly characterize such LNPs post modification. Figure 3c shows that the encapsulation efficiency for the prepared LNPs is generally greater than 95% and is not impacted by the chosen conjugation treatment with or without peptide modification. The only exception is the FAM-labeled peptide modification, which interferes with the underlying fluorescence measurement of the Ribogreen Assay.

Peptide quantification utilizing fluorophore labeled Peptide FAM-Cys-cRGD

4a Lipid Interference



4b Calibration Curve FAM-Cys-cRGD



4c FAM Assay

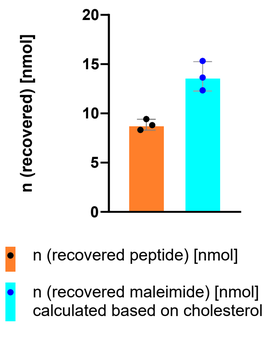


Figure 4 Peptide quantification with fluorophore-labeled peptides enables the quantification of low peptide densities with sufficient sensitivity and limited lipid interference, while monitoring the removal of excessive peptide by means of ultracentrifugation after in situ conjugation. In this case, cRGDfK was labeled with the fluorophore 5-carboxyfluorescein (5-FAM) and quantified based on a calibration standard of 5-FAM-Cys-cRGD. Figure 4a illustrates the lipid interference for quantification of 100 µL samples with a plate reader (Ex. 490 nm/Em. 520 nm) based on detected fluorescence intensity for PBS, Unmodified LNP control and FAM-Cys-cGRD-modified LNPs. Figure 4b describes the employed calibration standard for the quantification of peptide FAM-Cys-cRGD. Figure 4c Compares the amount of recovered peptide calculated using the FAM-Cys-cRGD calibration standard for peptide modified LNPs to the suspected amount of recovered click-reactive maleimide moieties available on the LNP sample surface post conjugation. The calculation is hereby based on the premise that there is no change in lipid composition during the formulation process, allowing for the calculation of available maleimide moieties based on determined cholesterol values considering the different percentages and using a cholesterol/PEG-maleimide ratio of 38.5/0.5 for the calculation.

Conclusion

This poster explores methods to enable the formulation and characterization of peptide-modified LNPs for tumor-targeted mRNA delivery utilizing active targeting strategies to selectively deliver its cargoe to tumor-associated endothelial cells and tumor tissue. Key challenges that are to be addressed are the accurate quantification of ligand density and comparability of targeting efficacy and transfection in vitro. RGD peptides as prototypic ligands were conjugated employing a thiol-maleimide click-reaction. For the purpose of custom mRNA generation an internal workflow was established that allows the cost-efficient generation of reporter and therapeutic constructs to investigate in vitro and in vivo applications of actively targeted peptide-modified LNPs. RGD peptide-modified LNPs are not impacted by the conjugation protocol in typical characteristics such as hydrodynamic diameter, PDI and encapsulation efficiency, unless a fluorophore-labeled peptide variant was employed. While fluorophore-labeled peptide-modified LNPs are unsuitable for standard particle characterization, they allow for reliable peptide quantification (ligand density), since this assay has a limited lipid interference and great sensitivity, enabling the quantification of low amounts of targeting ligands on the LNP surface.

Outlook

One of the key issues of peptide-modified LNPs is to identify a reliable peptide quantification method that allows for the quantification of ligand densities in a timely and routine manner. This problem necessitates assays for intra-formulation control, that do not entail a fluorophore labeling. While the shown FAM Assay excels in areas such as sensitivity and limited lipid interference, it interferes with routine methods for LNP characterization. Peptide quantification assays, such as the µBCA assay should be considered as attractive alternatives.

A schematic of alternative assays was assembled based on the premise of employing small, circular peptides such as the chosen cRGDfK peptide. A great choice for larger peptide structures is the CBQCA assay. As up to this point the listed assay alternatives could not produce congruent values for the peptide quantification of peptide-modified LNPs, in the future RPLC methods to quantify peptide will be the focus of further investigation.

	Arginine Assay	µBCA Assay (2% SDS)	CBQCA Assay	FAM-labeled Peptide
Concept	**Selective** derivatization of **arginine**	**Peptide-bond** induced reduction of Cu^{2+} ions and chelation of reagent	Derivatization of **primary amines** in proteins (e.g. lysine)	Quantification of **fluorophore-labeled peptide** after conjugation to LNPs
	Fluorescence	Absorbance	Fluorescence	Fluorescence
Sensitivity	ca. 2,5 - 40 µg/mL	0,5 – 20 / 2-40 µg/mL	0,25 - 50 µg/mL	-
Interference	Lipid	Lipid	-	-
Time	3 h + 1 h	1 - 2 h	1 h	0,5 h
cRGD compatibility	YES	YES	NO	YES

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